Methods for the Isolation and Identification of Salmonella, Listeria Monocytogenes, and Campylobacter Jejuni in Meat and Poultry Carcasses

Compiled by Jesse Ostroff
National Agricultural Library

September 1989

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This compilation comprises a collection of papers submitted by Dr. N. A. Cox, microbiologist, at the Food Protection and Processing Research Unit, Richard B. Russell Agricultural Research Center, U.S. Department of Agriculture, Athens, Georgia 30613, to the National Agricultural Library that represents laboratory methods for isolating and identifying Salmonella, Listeria monocytogenes, and Campylobacter jejuni--three types of organisms frequently encountered as causes of food poisoning in food and feed. The methods were designed primarily to assist meat and poultry inspectors as well as food sanitarians and commercial meat and poultry packing, slaughtering and processing plants throughout the world and represent the methods developed or adopted by the aforementioned laboratory to which Dr. Cox is attached. It should be borne in mind that the actual methods used by the Food and Safety Inspection Service (FSIS) can be obtained by writing to the Microbiology Division, Science, Food Safety Inspection Service, U.S. Department of Agriculture, South Building, Independence Avenue Between 12th and 14th Streets, S.W., Washington, D.C. 20250. FSIS is the U.S. Federal agency responsible for administering and enforcing the national Meat and Poultry Inspection Program.

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United States Department of Agriculture

Food Safety and Inspection Service

922693

14 JUN 1989

To: Microbiologists-in-Charge
Laboratory Directors

Through: R. A. Carnevale, Asst. Deputy Admr., SO

From: Ralph W. Johnston, Director
Microbiology Division, Science

Subject: Laboratory Communication No. 57 Revised May 24, 1989. Method for the Isolation and Identification of Listeria monocytogenes from Meat and Poultry products.

The method for testing products for the analysis of Listeria monocytogenes in meat and poultry products has been revised and is attached.

The major differences between this revision and the previous one are as follows:

- o Sample size is increased to 25 grams.
- o Samples collected under the agencies monitoring programs are described as are procedures for selecting 25 grams from these.
- o Fraser broth replaces the current secondary enrichment broth. It is a slight modification of the previously used UVM formula but permits more rapid reporting of negative samples and conservation of laboratory manpower and materials.
- o Plating agar has been changed to one that is slightly more selective. It also contains the esculin hydrolysis system which permits visual recognition of presumptive Listeria colonies, thereby eliminating the time consuming selection of colonies under the microscope and oblique light.

The FSIS strengthened Listeria policy is scheduled for implementation on July 1, 1989. Upon receipt of this L.C., purchase the necessary reagents and apply this procedure to the monitoring samples.

Under the new Listeria policy, a positive monitoring sample may result in the recall of the lots. The impact of this on the laboratories will be an increase in requests for results for both negative and positive samples.

Procedures for the detection of the L. monocytogenes are under development throughout the world and improvements are expected to be rapid. To keep pace with the progress, we expect to revise this method more frequently.

FSIS METHOD FOR THE ISOLATION AND IDENTIFICATION OF LISTERIA MONOCYTOGENES FROM PROCESSED MEAT AND POULTRY PRODUCTS

LABORATORY COMMUNICATION NO. 57
REVISED MAY 24, 1989

DENNIS MCCLAIN AND WEI HWA LEE

USDA, FSIS, MICROBIOLOGY DIVISION
BELTSVILLE, MD

INTRODUCTION

This method is intended for those with prior knowledge and experience in the isolation and identification of Listeria monocytogenes.

MEDIA AND REAGENTS

UVM BROTH (1); Primary Enrichment; Commercially Available (BBL;DIFCO)

This medium is essentially the medium that was developed by Donnelly and Baigent at the University of Vermont (2). It differs from the original formula in that it contains one-half the amount of naladixic acid.

Proteose Peptone	5	g
Tryptone	5	g
Lab Lemco Powder	5	g
Yeast Extract	5	g
NaCl	20	g
KH_2PO_4	1.35	g
Na_2HPO_4	12	g
Esculin	1	g
Naladixic Acid (2% in 0.1 M NaOH)	1	ml
Acriflavin	12	mg
Distilled Water	1	liter

Sterilize at 121°C, 15 minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. IF THE MEDIA BLACKENS OR DARKENS, IT HAS BEEN OVERHEATED AND MUST BE DISCARDED. Store in the refrigerator.

FRASER BROTH; Secondary Enrichment (3)

This broth is identical in formula to that above except for increased acriflavin to aid in selection and the addition of lithium chloride and ferric ammonium citrate to produce a visual blackening of tubes containing esculin hydrolyzing bacteria. All Listeria species, and other bacteria that hydrolyze esculin, darken or blacken this medium.

Proteose Peptone	5	g
Tryptone	5	g
Lab Lemco Powder	5	g
Yeast Extract	5	g
NaCl	20	g
KH_2PO_4	1.35	g
Na_2HPO_4	12	g
Esculin	1	g
Naladixic Acid (2% in 0.1 M NaOH)	1	ml
Lithium Chloride	3	g
Distilled Water	1	liter

Mix well to resuspend the media and dispense 10 ml into 20 x 150 mm test tubes. Sterilize at 121°C, 15 minutes. DO NOT OVERHEAT; COOL AT ONCE AFTER REMOVAL FROM THE STERILIZER. Store in the refrigerator. Just before use, add 0.1 ml of 2.5 mg/ml of filter sterilized acriflavin (Sigma) and 0.1 ml filter sterilized 5% stock solution of ferric ammonium citrate (Sigma) in distilled water to each 10 ml tube.

MODIFIED OXFORD MEDIUM (MOX)

MOX agar is a slight modification of the Oxford Listeria selective medium developed by Curtis et al. (4).

MOX Agar Base:

Columbia Blood Agar Base	39-44	g/l (depending on brand)
Agar	2	g/l
Esculin	1	g/l
Ferric Ammonium Citrate	0.5	g/l
Lithium Chloride (Sigma L0505)	15	g/l
1% Colistin Solution	1	ml
Distilled Water	1000	ml

Rehydrate with constant stirring with a magnetic mixer and adjust pH to 7.2 if necessary. Autoclave this base at 121°C for 10 minutes, mix again, and cool rapidly to 46°C in a water bath. Add 2 ml of 1% filter sterilized Moxalactam Solution to make the complete MOX medium, mix well, and pour 12 ml per plate.

CAUTION: Do not use the Oxford supplement or any other supplement with this formula.

1% Colistin Solution:

Colistin, Methane Sulfonate (Sigma C1511)	1	g
0.1 M Potassium Phosphate Buffer, pH 6.0	100	ml

Colistin solution is not sterile, store frozen in small aliquots (3-5 ml) at -20°C or below.

1% Moxalactam Solution:

Sodium (or Ammonium) Moxolactam (Sigma M1900)	1	g
0.1 M Potassium Phosphate Buffer, pH 6.0	100	ml

Dissolve, sterilize by filtration, dispense in 2 ml quantities and store in freezer (-20°C or below).

HORSE BLOOD OVERLAY MEDIUM (HL)

Base Layer:

Columbia Blood Agar Base 1 liter; prepared to manufacturers specifications.

Sterilize at 121°C for 15 minutes. Pour 10 ml per 100 mm diameter petri dish. Allow to solidify and while still warm, overlay with blood agar as described below.

Top Layer:

Add 4% horse blood to melted Columbia blood agar base, which has been cooled to 46°C. Stir or swirl to mix evenly. Put 5 or 6 ml on top of the base layer and tilt the plates to spread top layer evenly. Store plates in the refrigerator. Discard any discolored plates. THIN PLATES ARE NECESSARY TO DEMONSTRATE LISTERIA BETA-HEMOLYSIS PRODUCED BY SURFACE COLONIES.

CAMP TEST AGAR

Use Trypticase Soy Agar containing 5% sheep blood. Pour 8 ml per 100 mm diameter petri dish. Store plates in the refrigerator. Discard any discolored plates.

CARBOHYDRATE FERMENTATION BROTH

Base:

Peptone	10	g
Lab Lemco Powder (OXOID)	1	g
NaCl	5	g
Distilled Water	900	ml
Phenol Red (360 mg/20 ml 0.1 N NaOH)	1	ml

Adjust pH to 7.4 and sterilize at 121°C for 15 minutes. To the cooled basal fermentation broth, add 100 ml of filter sterilized carbohydrate solutions as indicated below:

Xylose, 5%
Mannitol, 10%
Rhamnose, 5%

Dispense aseptically: 5 ml per 13 x 100 mm sterile screw cap tubes.

PROCEDURE

SAMPLE SIZE

For products collected under the Agency's various monitoring or follow-up programs, a 25 gram portion will be selected as described under sample preparation. In illness or potential illness, investigations may result in larger sample sizes.

SAMPLE PREPARATION

Sanitize the surface of the intact meat package by either of the following procedures:

(a) Swabbing it with 3% Hydrogen Peroxide. Use peroxide from the drug store and add one teaspoonful of Johnson's NO MORE TEARS baby shampoo (or equivalent) to a 1 pint bottle. The shampoo named was chosen because it is neutral and contains EDTA which stabilizes peroxide. Rotate the bottle gently to mix the contents. Allow the peroxide to dry on the package before proceeding.

(b) Dipping the package into a bleach solution. This solution is made by adding 50 ml of household bleach and 0.5 g Tween 80 to 1000 ml of 0.003 M NaH_2PO_4 buffer and adjusting the pH to 6.0. Allow the bleach to air-dry on the package. Open the package using sterile knives, scissors, and forceps.

The following media are obtainable in dehydrated form and are made as per manufacturer's directions:

BACTO MOTILITY TEST MEDIUM (Difco 0105-01-3); CAUTION: Many similar motility preparations do not give the typical umbrellaform motility. Dispense 4 ml per 13 x 100 test tubes.

BRAIN HEART INFUSION BROTH (BHI)

BRAIN HEART INFUSION AGAR SLANTS

BILE ESCULIN AGAR SLANTS

MR-VP MEDIUM

OXIDATION/FERMENTATION MEDIUM (O/F)

NITRATE BROTH

OXIDASE TEST REAGENT (1% tetramethyl-p-phenylenediamine dihydrochloride)

THE MEDIA CONTAINED IN THIS SECTION ARE, ON THE WHOLE, HEAT-SENSITIVE, AND IT IS IMPORTANT TO OBSERVE THE TIMES AND TEMPERATURES GIVEN FOR THEIR PREPARATION. LEFT-OVER, UNPOURED AGAR SHOULD BE DISCARDED.

A. Monitoring Samples:

Under the revised instructions for the Listeria monitoring program, the inspector will be collecting five (5) intact retail packages of a product if the product is packaged for retail in sizes of 1 pound or less. If the product is packaged in sizes of greater than 1 pound or bulk packaged, the inspector will aseptically collect a single representative portion. The laboratory procedures will be different for these two cases.

1. Packaged product of 1 pound or less:

   a. Inspector will collect five (5) retail packages from one lot.

   b. Laboratory disinfects packages and removes a 5 gram representative portion from each package into a blender or Stomacher container to comprise one composite sample of 25 grams for a lot.

2. Bulk packaged, packaged product of greater than 1 pound or product arriving in the laboratory not in a retail package:

   a. Inspector will collect one representative portion from one lot (e.g., center slice of roast beef).

   b. Laboratory removes a 25 gram representative portion into a blender or Stomacher container to comprise one 25 gram sample for a lot.

B. Refrigerated Shelf Life Sample:

1. In addition to the five retail packages of 1 pound or less, the inspector will also be collecting one sample to be analyzed at the end of shelf life.

2. The laboratory will refrigerate this sample (must be intact) at 40-42°F until the end of shelf life. The tracking system for this will be developed by the laboratories. If no shelf life is indicated, the laboratory must visually inspect the package periodically for visual indication (e.g., milkiness) which indicates end of acceptable shelf life. If no indication by 70 days post production, the laboratory may analyze the shelf life sample. If the shelf life is known, the sample analysis should be scheduled for analysis just after the end of the "use by" date. If the sample has no "use by" date but has a "sell by" date, analyse just after the end of the "sell by" date. Incubator temperatures should be continuously recorded.

   The laboratory will sanitize package and remove a 25 gram representative portion into a blender or Stomacher container.

ENRICHMENT AND PLATING

Add 225 ml of Listeria enrichment broth (UVM) to each 25 g sample. Blend or stomach for 2 minutes. Transfer blended material to a suitable sterile container and incubate for 20-24 hours at 30°C. The Stomacher bag may be sealed with a small air pocket and incubated without transfer.

Secondary Enrichment:

Transfer 0.1 ml of the incubated broth to Fraser broth. Incubate at 35°C for 26 +/- 2 hours. Compare each incubated tube to an uninoculated tube against a white background. Blackened or darkened tubes resulting from esculin hydrolysis are to be streaked for isolation. Culture tubes that remain the original straw color are to be reported as negative for L. monocytogenes.

Dip a sterile cotton swab into the Fraser broth positive tubes and swab 1/2 of a MOX agar plate. With a loop, streak the remainder of the plate at a 90° angle twice (Fig. 1). Incubate the plate at 35°C for 24-48 hours.

NOTE: When testing samples suspected for involvement in illness complaints, streak MOX plates from the Fraser broth regardless of color reaction, incubate the Fraser broth an additional 24 hours then restreak to MOX agar plate. Incubate this plate at 35°C and read at both 24 and 48 hours.

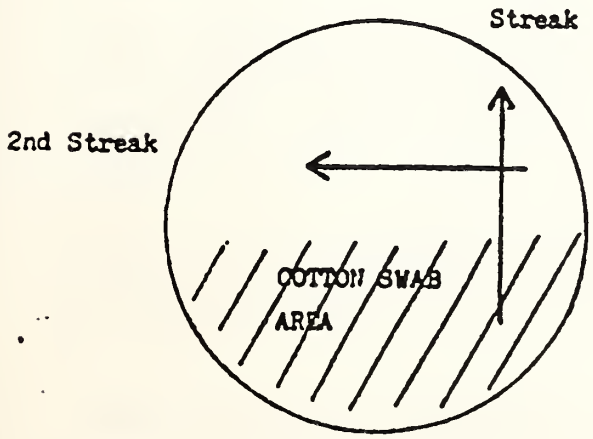

Fig. 1: Swab and streaking patterns for Mox agar.

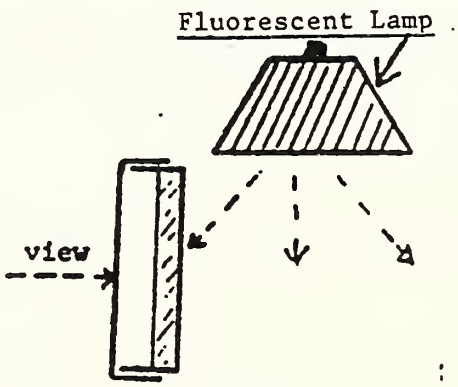

Fig. 2: Viewing angle for horse blood overlay plates.

EXAMINATION OF INCUBATED MOX AGAR PLATES

MOX agar is highly selective and yields Listeria monocytogenes colonies typical in size and formation. Esculin is hydrolyzed resulting in black zones around the colonies. Typical L. monocytogenes colonies are almost always visible after 24 hours, but incubation should be continued for an additional 24 hours to detect slow growing strains.

COLONY SELECTION AND RESTREAKING

Gently touch five colonies suspected of being Listeria in succession with the inoculation needle. Streak for isolation onto a Horse Blood Overlay Agar plate. This should be done for each plate inoculated the previous day. Because the plates are so thin they are easily scratched and should be carefully streaked with a pure platinum wire. Incubate overnight at 35°C.

EXAMINATION OF INCUBATED HORSE BLOOD OVERLAY AGAR PLATES

Examine the plates with a fluorescent lamp. Hold the plate at a right angle to a fluorescent lamp and view the bottom of the plate (Fig. 2). Select translucent colonies, about 1-2 mm in diameter, with a narrow zone of beta hemolysis surrounding the colony and complete clearing of the blood underneath.

THIN PLATES AND A FLUORESCENT LAMP ARE NEEDED TO DEMONSTRATE BETA HEMOLYSIS BY SURFACE COLONIES OF Listeria monocytogenes.

SCREENING

Well isolated colonies suspected of being Listeria monocytogenes are picked with a needle and transferred to BHI Broth and to Bacto Motility Test Medium by stabbing. Incubate both media at 20-25°C. After overnight incubation, examine BHI broth by wet mount or hanging drop for tumbling motility; also make a gram stain.

NOTE: Proceed with identification tests only if the culture is hemolytic gram-positive, has tumbling motility, and appears to be a pure culture. Consult the flowchart (Fig. 3) and Table 1 for aid in the evaluation of the screening tests performed.

IDENTIFICATION AND CONFIRMATION TESTS

Media used in the identification and confirmation of Listeria monocytogenes can be inoculated from the BHI broth. Inoculate a slant of BHI agar to be used for the catalase and oxidase tests.

Also inoculate bile esculin agar, MR-VP medium, O/F medium, nitrate broth, and rhamnose, xylose, and mannitol fermentation broths.

Fig. 3: FSIS Identification Schema for the Isolation of Listeria Monocytogenes

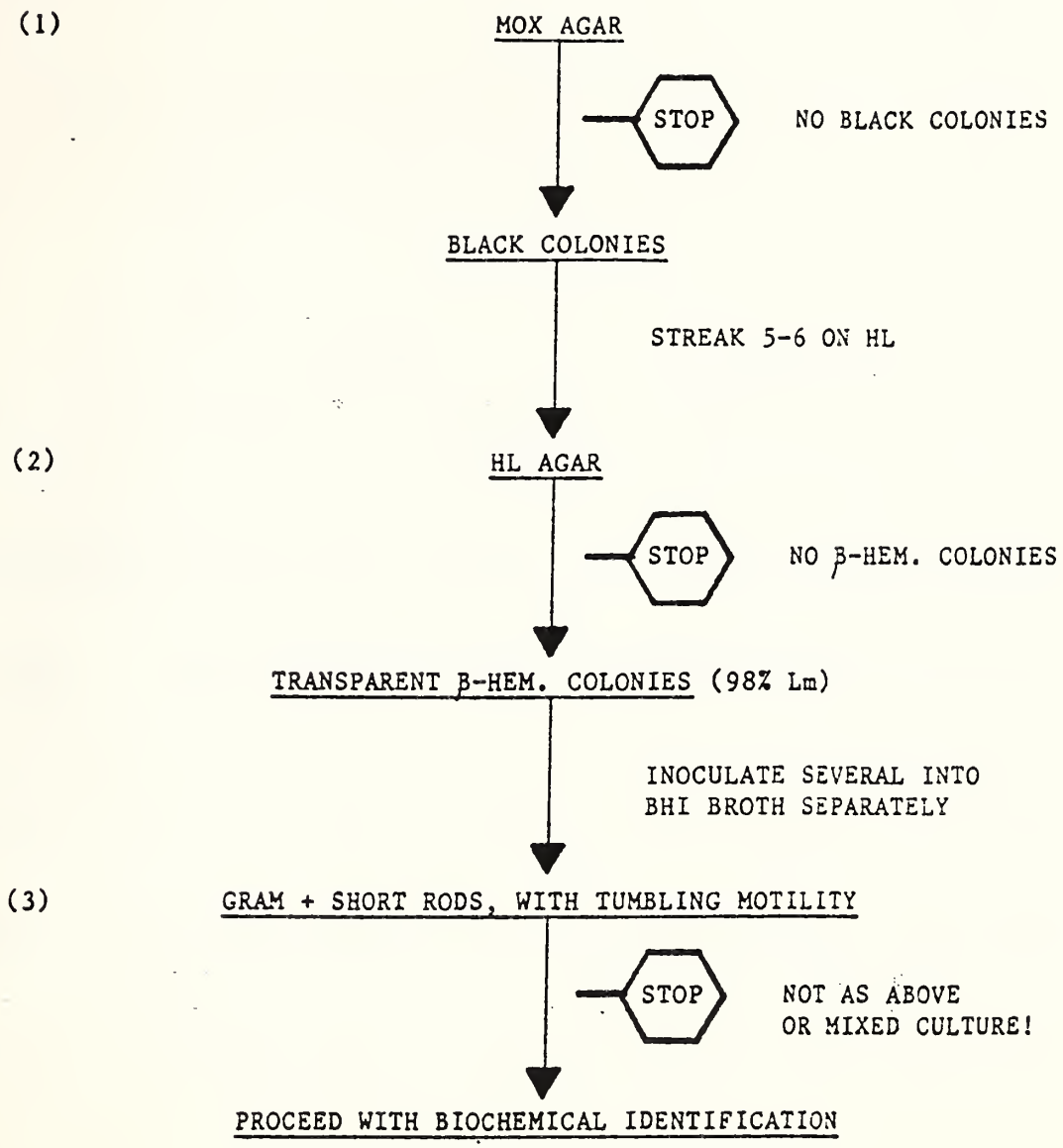

Table 1 . Reactions for Screening Listeria Species.*

	Reactions				
	HL Agar		Motilty **		
Species	Colony	Hemolysis	Agar	Broth	Stain
L. monocytogenes	Transparent	beta	Umbrella-like	Tumbling	Gram +
L. seeligeri	Transparent	beta	Umbrella-like	Tumbling	Gram +
L. ivanovii	Transparent	beta	Umbrella-like	Tumbling	Gram +
L. innocua	Transparent	no	Umbrella-like	Tumbling	Gram +
L. welshimeri	Transparent	no	Umbrella-like	Tumbling	Gram +

* Manual of Clinical Microbiology, 1988

** Since hemolysis reaction occurs before final reading of motility, discontinue motility tests for all non-hemolytic isolates.

Incubate the motility medium for 2 or more days. Observe for an umbrella type of growth (Fig. 4) which is typical of Listeria monocytogenes. Discard cultures which lack this appearance.

Fig. 4: Umbrella Motility

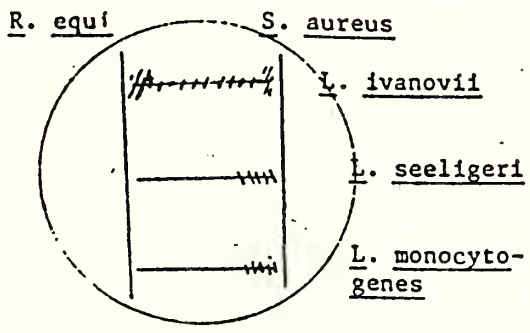

Fig. 5: CAMP Test

MODIFIED CAMP TEST (1)

Inoculate a line of fresh BHI broth cultures of Staphylococcus aureus, ATCC 25923 and Rhodococcus equi, (FDA Strain) on a plate of the test agar. Streak suspected listeriae at a 90° angle (Fig. 5). Incubate the plates at 35°C for 24-48 hours. A positive reaction is an arrow shaped hemolytic zone close to the Staphylococcal or Rhodococcal growth. L. monocytogenes and L. seeligeri are CAMP positive to the Staphylococcus and CAMP negative to the Rhodococcus while L. ivanovii is CAMP positive to both the Staphylococcal and the Rhodococcal growth.

Alternative CAMP Test (1):

A more convenient CAMP test may be performed using the S. aureus factor in commercially prepared sterile β-lysin discs (Remel □21-120). In this test, a β-lysin disc is placed in the center of the 8 ml CAMP sheep blood plate and 4-5 Listeria cultures are streaked as radiating lines from the disc. After overnight incubation at 35°C, a very sharp CAMP reaction between L. monocytogenes or L. seeligeri cultures and the disc can be observed. L. ivanovii are strongly hemolytic and forms a clear B-hemolytic line along the entire streak. It is also xylose positive.

BASIC IDENTIFICATION CHARACTERISTICS OF L. MONOCYTOGENES

Listeria monocytogenes is a gram-positive, non-sporeforming short rod that exhibits tumbling motility at 25°C. It is beta-hemolytic and gives a positive CAMP test with S. aureus, but not R. equi, on sheep blood agar. It is catalase positive, oxidase negative, MR and VP positive, bile esculin positive, fermentative with respect to glucose, and rhamnose, fails to ferment xylose and mannitol and does not reduce nitrate. See Table 2 for aid in the identification of L. monocytogenes and other Listeria.

Table 2. Confirmatory Tests for Hemolytic Listeria

Species	Catalase	Oxidase	Bile Esculin	MR - VP	Glucose O - F	CAMP Staph.aureus	CAMP R. equi	Nitrate Reduction	Xylose	Rhamnose	Mannitol
L. monocytogenes	+	-	+	+ / +	+	+	-	-	-	+	-
L. seeligeri	+	-	+	+ / +	+	+	-	-	+	-	-
L. ivanovii	+	-	+	+ / +	+	+	+	-	+	-	-

REFERENCES

(1) McClain, D. and Lee, W. H. 1988. JAOAC. 71:660-664.

(2) Donnelly, C. W. and G. J. Baigent. 1986. Appl. Microbiol. 52:684-695.

(3) Fraser, J. A. and Sperber, W. H. 1988. J. Food Prot. 51:762-765.

(4) Curtis, G. D. W., Mitchell, R. G., King, A. F. and Griffin, E. J. 1989. Appl. Microbial. 8:95-98.

Concurrence:

Wei Hwa Lee Acting — 5/24/89

W. H. Lee, Acting Chief, FMB, MD, SCI — Date

Ralph W. Johnston — 6-14-89

R. W. Johnston, Director, MD, SCI — Date

Reprinted from
Journal of Food Protection, Vol. 51, No. 5, Pages 391-396 (May, 1988)

MLIA/USDA

Improved Selective Procedure for Detection of Salmonellae from Poultry and Sausage Products

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(Received for publication October 1, 1987)

ABSTRACT

The efficacy of lactose preenrichment and various selective enrichment and differential plating media were evaluated to determine the optimal procedure for detecting salmonellae from fresh chicken or frozen turkey, pork sausage, and cured chicken. Salmonellae were most frequently recovered from fresh poultry or pork sausage when samples were preenriched in lactose broth incubated at 35°C, selectively enriched in TT broth at 43°C, and streaked onto a new differential plating medium, modified lysine iron agar (MLIA/USDA). Enrichment of cured chicken in selenite brilliant green broth incubated at 43°C was more productive than in TT incubated at 43°C. When poultry and sausage samples were first preenriched at 35°C, selectively enriched at 43°C, and then streaked onto MLIA/USDA greater than 75% of all CFUs on the MLIA/USDA plates were typical of salmonellae. Different procedures are recommended for maximal recovery of salmonellae from fresh, frozen or cured poultry products.

The FDA and USDA official methods for isolation of *Salmonella* from raw meats are inconsistent. The FDA procedure calls for direct enrichment in selenite cystine (SC) and tetrathionate brilliant green (TBG) for 24 h at 35°C followed by streaking on selective plates of bismuth sulfite (BIS), xylose lysine desoxycholate (XLD), and Hektoen enteric (HE) agars which are incubated for 24 h at 35°C *(1)*. The USDA procedure calls for preenriching the meat samples in lactose broth (LB) with 0.6% tergitol for 24 h, then transferring 0.5 ml of the LB to 10 ml of TT *(16)* broth which is incubated for 24 h at 35°C. Selective plates of brilliant green sulfa (BGS) and XLD agars are then streaked and incubated for 24 h at 35°C *(27)*.

It is generally recognized that to detect sublethally injured salmonellae from processed foods it is necessary to preenrich the food product in a non-selective broth. This procedure has not been universally accepted for raw meat products which have not undergone processing steps which would lead to sublethal injury of contaminating bacteria. In addition to the recommendation of direct enrichment by FDA, van Schothorst et al. *(28)*, Cox and Mercuri *(3)*, and Cox et al. *(5)* have also used direct incubation of raw, chilled or frozen meat products in selective enrichment media. Conversely, in addition to USDA using a non-selective preenrichment, Galton et al. *(14)*, Surkiewicz et al. *(25)*, Gabis and Silliker *(13)*, and many other have suggested that more complete recovery of *Salmonella* from raw products is achieved when a non-selective preenrichment broth is used before selective enrichment. Based on these findings, and since the USDA studies already recommended its use, it was decided to use preenrichment in most of our meat samples in this study.

D'Aoust and Maishment *(7)* evaluated six non-selective preenrichment media, and found all of them except EE broth *(19)* to be equally effective for isolation of *Salmonella* in naturally contaminated low and high moisture foods. They also found that *Salmonella* could be recovered from significantly more samples preenriched for 24 h than when they were preenriched for only 6 h. Because of its widespread use in the U.S.A. and apparent effectiveness with meat products, LB preenrichment for 24 h was chosen for this study.

Tetrathionate broth or some modification (e.g. TBG or TT) and SC are the most widely used of the numerous selective media for isolation of salmonellae from foods and maximal recovery can be obtained by using a combination of tetrathionate broth and SC *(2,10,20)*. In addition to TT and SC, selenite brilliant green (SBG) broth *(24)* was tested as one of our primary selective enrichment media. Previous studies *(3,5)* in our laboratory have demonstrated that for raw processed poultry, direct enrichment with SC would detect as few as four cells of *Salmonella* per broiler carcass. Therefore, the sensitivity of direct enrichment of meat samples in SC incubated at 35°C was examined.

Both the FDA and USDA recommend incubating preenrichment and selective enrichment media at 35°C. However, the international organizations, ICMSF and ISO, recommend incubating of preenrichment at 35°C and enrichment media at 43°C. Other studies with both clinical and food samples have shown that incubation of cultures at 43°C will inhibit growth of other *Enterobacteriaceae* but not *Salmonella*. Silliker and Gabis *(23)* found more *Salmonella* to be detected when frozen raw meat samples were preenriched at 35°C

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followed by incubation of enrichment cultures at 43°C than if the enrichment cultures were incubated at 35°C. In this study, plates were streaked from samples preenriched in lactose broth incubated at 35 and 43°C and from TT or SBG enrichment cultures incubated at 43°C obtained by subculturing from the lactose broth incubated at 35°C.

Several differential plating media are recommended for isolation of salmonellae. In the United States, BGS, XLD, HE and BIS are the most commonly used plating media. In this study, HE was not chosen because many meat and poultry samples will contain strains of *Proteus* which look the same as *Salmonella* on the HE plates and extra work will be required to differentiate these false-positive isolates. Also, BIS was not used because the selectivity and differential properties can change dramatically as a result of preparation and storage conditions *(6)*, and the wide divergence in appearance of *Salmonella* colonies on these plates also requiring extra work to confirm positive *Salmonella* samples. In this study, we used BGS, XLD with and without added novobiocin which inhibits growth of *Proteus (17,22)*, and a variation of the modified lysine iron agar of Rappold and Bolderdijk *(21)* which was changed to enhance the detection of H_2S.

The type of food products being tested and the types of competing microorganisms will affect the performance of *Salmonella* recovery media suggesting that an optimal procedure should be determined for each different food category *(2,10)*. The objective of this study was to determine optimal media and conditions for recovery and isolation of *Salmonella* from fresh and cured raw meat and poultry products.

MATERIALS AND METHODS

Experiment 1

Twenty-five 1-lb samples of mechanically deboned turkey (MDT) were collected over a period of 2 weeks from a commercial plant. The samples were frozen in the commercial freezer at each processing plant and shipped to the laboratory where they were sampled within 1 month. Each sample was thawed at room temperature and sampled in the following manner (Fig. 1). Twenty-five g was placed in 225 ml of lactose broth with 0.6% tergitol, stomached for 30 s, and incubated at 35°C for 24 h. A duplicate 25-g sample was preenriched in lactose broth incubated at 43°C for 24 h. One-ml portions of the lactose broth which had been incubated at 35°C was inoculated into 9 ml of each of three enrichment media: *(a)* TT broth (Difco) incubated at 35°C for 24 h, *(b)* TT broth incubated at 43°C for 24 h, and *(c)* selenite brilliant green (SBG) broth (Difco) incubated at 43°C for 24 h. In addition, 25 g of the thawed turkey sample was placed in 225 ml of selenite cystine (SC) broth (Difco) and incubated at 35°C for 24 h.

After incubation of all samples, one loopful (3-mm loop) of each broth was streaked onto BGS, XLD, and XLD plus 10 mg of novobiocin/L (XLD-N) plates. Two typical colonies were picked from each plate and inoculated into lysine iron (LI) agar slants. Each culture showing presumptive-positive LI results were serologically tested (poly O, subgroup O) and confirmed (poly H) to be *Salmonella*. Twenty-five 1-lb samples of mechanically deboned cured chicken (MDCC) meat and 25 1-lb samples of fresh pork sausage were each collected from single commercial plants and all samples were analyzed as previously described for MDT.

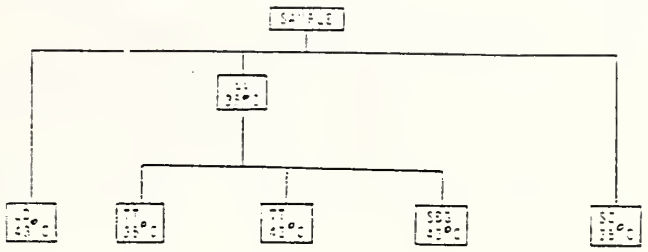

Figure 1. *Sampling scheme for fresh and cured meat samples (Experiment 1): LB-lactose broth, TT tetrathionate brilliant green (16), SBG-selenite brilliant green, SC-selenite cystine.*

Experiment 2

Fifty 1-lb samples of MDT were collected as described in Experiment 1. After thawing, 25 g of each sample was placed in 225 ml (SC), stomached for 30 s, then incubated for 24 h at 35°C. In addition, 25 g of each sample was placed in 225 ml of lactose broth with 0.6% tergitol and stomached for 30 s. After incubation for 24 h at 35°C, replicate 1-ml portions of the lactose preenrichment culture were inoculated into 9 ml of TT and SBG and incubated for 24 h at 43°C. One ml from each enrichment culture were then combined and immediately inoculated onto BGS, XLD-N and MLIA (Table 1). Suspect colonies were screened biochemically and serologically as previously described.

Fifty 1-lb samples of MDCC were obtained commercially and treated in the same manner as MDT. Also, 50 processed broiler carcasses were obtained from a poultry processing plant and transported to the laboratory in ice. Carcasses were rinse-sampled according to the procedure of Cox et al. *(4)*, except that 300 ml of sterile rinse water was used. After rinsing, the remaining 210 ml of rinse fluid was split into two equal volumes and concentrated SC (10x) or lactose broth (10x) were added to bring the sample to a single strength solution. Duplicate 1-ml portions of the lactose preenrichment culture were inoculated into 9 ml of TT and SBG and incubated for 24 h at 43°C. Enrichment cultures were streaked on BGS, XLD-N and MLIA. Thereafter, the samples were treated the same as the others in Experiment 2. Also, 0.5 ml of the TT (43) and 0.5 ml of the SBG (43) enrichment broths were combined and the composite was streaked onto the appropriate selective plating media. Data were analyzed by ANOVA and the Duncan Multiple Range Test *(8)*.

TABLE 1. *MLIA/USDA.*

Lysine iron agar (Oxoid)	34 g
Bile salts No. 3 (Oxoid)	1.5 g
Lactose	10 g
Sucrose	10 g
Sodium novobiocin	.015 g
Sodium thiosulfate	6.8 g
Ferric ammonium citrate	0.3 g
Distilled water	1000 ml

TABLE 2. *Comparison of enrichment and plating media for recovery of* Salmonella *from mechanically deboned turkey (MDT), mechanically deboned cured chicken (MDCC), and pork sausage (Experiment 1).*

		No. of samples confirmed *Salmonella* (+) out of 25 samples						
		Preenrichment[b]		Enrichment[b]				
Product	Media	LB (43)	LB (35)	TT (35)[c]	TT (43)[c]	SBG (43)[c]	SC (35)	Total[d]
Turkey	BGS	1	0	2	13	0	1	13
	XLD	1	0	3	12	0	1	
	XLD-N	2	0	4	12	0	1	
Cured chicken	BGS	21	9	20	15	22	3	
	XLD	12	3	5	2	21	1	22
	XLD-N	9	2	17	13	22	3	
Pork sausage	BGS	0	0	0	2	0	0	
	XLD	0	0	0	2	0	0	2
	XLD-N	0	0	0	2	0	0	

[a]BGS - brilliant green sulfa, XLD - xylose lysine desoxycholate, XLD-N - XLD + 10 mg/L novobiocin.
[b]LB - lactose broth, TT (Hajna and Damon, 1956), SBG - selenite brilliant green, SC - selenite cystine, (35) - 35°C incubation, (43) - 43°C incubation.
[c]LB (35) preenrichment broth used to inoculate TT (35), TT (43) and SBG (43) selective enrichment.
[d]Total positive samples by one or more methods.

TABLE 3. *False-positives on differential plating media samples of deboned turkey and cured chicken meat.*

Plating media	Number suspect colonies	False-positives	
		Number	Percent
BGS	264	5	1.9
XLD	271	144	53.1
XLD-N	258	34	13.2

RESULTS

Experiment 1

With MDT samples (Table 2), direct streaking from LB preenrichment or SC enrichment samples was not effective for recovering salmonellae. When LB (35) preenrichment was transferred to the different enrichment media, more salmonellae were recovered from the TT samples than the SBG samples, and the TT samples incubated at 43°C yielded more positives than the TT samples incubated at 35°C. Productivity of plating media were comparable. The most salmonellae were recovered from MDCC when samples were streaked from SBG (43) onto any of the plating media or when streaked onto BGS plates from TT (35) or LB (43) (Table 1). With the MDCC, TT (43) was not as effective as TT (35) and BGS was the most effective plating medium. With pork sausage, salmonellae were only recovered from two samples and only when samples were incubated in TT (43) following enrichment in LB (35). The two salmonellae recovered were detected on all three plating media.

The total number of turkey and cured chicken samples from which salmonellae were detected was about the same for the BGS, XLD or XLD-N plates, but 53.1% of the salmonellae-like isolates selected from XLD plates were not salmonellae. The occurrence of false-positive results varied with plating media. When novobiocin was added to the XLD plates, the number of false-positives was reduced to 13.2% while only 1.9% of the isolates from BGS were needlessly picked (Table 3).

Experiment 2

There were significantly more recoveries of salmonellae from MDT samples (Table 4) using TT (43) or the composite TT (43) and SBG (43) than from SC (35) or SBG (43), but no significant differences among plating media were observed. The number of salmonellae recoveries from cured chicken was similar for the four enrichment procedures, but significantly more salmonellae were recovered with BGS and MLIA/USDA than with XLD-N. With chicken rinse, there was no significant difference in the number of salmonellae recovered using the TT (43), SC (37) or the composite TT (43) and SBG (43) and all three enrichments gave significantly ($P = <.05$) more recoveries than the SBG (Table 4). There were no significant differences in plating media although MLIA/USDA and XLD-N gave more positive recoveries than BGS.

Overall, salmonellae were recovered significantly ($P = <.05$) more frequently with TT (43) or the composite of TT (43) and SBG (43) than with SBG (43) alone or from direct enrichment in SC (37). Salmonellae were more frequently isolated with MLIA/USDA than from BGS or XLD-N. With one or more of the procedures used, salmonellae were found in 92% of the cured chicken samples, 72% of the turkey samples, and 98% of the chicken carcasses. The lowest percentage of false-positive results were obtained with MLIA following preenrichment in lactose at 35°C and enrichment in TT at 43°C (Table 5).

DISCUSSION

When determining whether to directly enrich a sample or to use a non-selective preenrichment step first, it is important to know the expected number of *Salmonella* present and their

TABLE 4. *Comparison of enrichment and plating media for recovery of* Salmonella *from turkey, deboned cured chicken and broiler carcasses (Experiment 2).*

Product	Plating media	No. of samples confirmed *Salmonella* (+) out of 50 samples				
		TT (43)[a]	SBG (43)[a]	TT (43)[a] + SBG (43)	SC (37)[b]	Total[c]
Turkey	MLIA/USDA	34	20	35	24	
	BGS	35	6	28	27	36
	XLD-N	35	7	36	28	
Cured chicken	MLIA/USDA	37	39	45	29	
	BGS	37	34	36	28	46
	XLD-N	27	32	30	28	
Chicken rinse	MLIA/USDA	49	40	49	45	
	BGS	49	19	37	38	49
	XLD/N	49	26	48	49	

[a]9-ml TT or SBG enrichment culture inoculated with 1 ml of lactose preenrichment broth incubated at 35°C.
[b]Samples were directly enriched in SC with no enrichment.
[c]Total positive samples by one or more methods.

TABLE 5. *Selectivity of MLIA/USDA agar medium.*

Product sampled	Enrichment broth			
	SC (37)	TT (43)	SBG (43)	TT+SBG (43)
Deboned turkey meat	30[a]	95	45	60
Deboned cured chicken meat	50	75	55	55
Broiler carcass rinse	45	95	45	65

[a]Number of confirmed colonies (%).

physiological state as well as knowing the type and number of background flora which are present in the sample being tested. Large populations of physiologically active salmonellae will facilitate detection and many selective recovery media can be used to detect the salmonellae, but if only a few organisms are present, if the salmonellae are in an inactive state, or if the meat sample being tested is highly contaminated with competitive flora, then direct enrichment of samples without prior preenrichment is not justified and may lead to false-negative results.

Tetrathionate-based enrichment media are very selective and can even be somewhat toxic to salmonellae. Taylor and Silliker *(26)* and Bailey et al. *(2)* showed that detection of low levels of *Salmonella* from various food products was not always achieved when the foods were directly inoculated into tetrathionate-based broths. If the number of *Salmonella* in these products was increased either by increasing the size of inoculum or by preenriching in a non-selective medium, then *Salmonella* were detected in all samples and in some instances the tetrathionate media detected the *Salmonella* when the less selective selenite cystine did not. In these instances where the initial numbers and condition of the *Salmonella* were sufficient to survive the initial shock of the enrichment media, the tetrathionate medium was more effective because the competing background microflora was suppressed while the *Salmonella* were able to grow to sufficient numbers to be detected.

Numerous studies have shown that if salmonellae are not injured, most of them will grow at 43°C. At the same time, many competing bacteria are inhibited at this temperature. The selective properties of TT broth are, therefore, enhanced by incubating the samples at 43°C. In Experiment 1 of this study, we also found that more salmonellae recoveries were made from fresh turkey and pork sausage when the samples were preenriched at 35°C and subsequently transferred to TT broth which was incubated at 43°C than when TT broth was incubated at 35°C. In addition to the salmonellae being recovered from more samples, a higher percentage of the isolates on the selective plates were confirmed to be salmonellae when TT (43) was used (Table 5). With the fresh products tested, 95% of all CFUs were confirmed to be salmonellae.

With the cured chicken meat which contained about 2.0 to 3.0% added NaCl and about 0.15 to 0.2% added $NaNO_2$, incubation of the samples in TT (43) was less effective for recovery of salmonellae than incubation at TT (35). Different media and methods are necessary for maximal recovery of salmonellae from fresh or cured meats and a possible explanation of these differences would be the added NaCl and nitrites. Many bacteria are inhibited by the levels of added solutes used and the combination of the solutes, elevated incubation temperature (43°C) and TT enrichment broth seemed to have a detrimental effect on recovery of salmonellae. The most effective method for recovery of salmonellae from cured chicken meat was with the less selective SBG (43) enrichment (Tables 2 and 4). In Experiment 2, favorable recovery of salmonellae was also achieved with TT (43) enrichment followed by streaking onto MLIA/USDA or BGS plates (Table 4), but no comparison was made to TT (35).

With the exception of BIS, most selective plating media for isolation and differentiation of *Salmonella* have used lactose fermentation as the primary biochemical reaction to differentiate the *Salmonella* from the other bacteria capable of surviving selective enrichment. The genus *Salmonella* was subdivided into 5 subgroups in 1984 *(11)*. *Salmonella* subgroup 1 are usually isolated from humans and warm-blooded animals. *Salmonella* subgroup 3 (formerly *Arizona*) are usu-

ally isolated from cold-blooded animals and the environment. Strains belonging to subgroup 3 have been found in a variety of animal sources *(9)* including poultry *(15)*. Only 1% of subgroup 1 isolates are reported to be lactose fermenters while 15% of subgroup 3a (monophasic arizonae) and 85% of subgroup 3b (diphasic arizonae) ferment lactose *(12)*. Detection of the subgroup 3 isolates based on lactose fermentaqtion could, therefroe, be a problem, but this is not usually true because many subgroups 3 isolates are slow lactose fermenters, normally taking more than 72 h to ferment lactose. Therefore, for poultry and meat products it should not be necessary to use BIS selective plates.

In Experiment 1, the total number of fresh turkey and sausage samples found to be positive for salmonellae was about the same for the BGS, XLD or XLD-N plating media, but the prevalence of suspect colonies was greater when novobiocin was added to the XLD, which confirmed the work of Moats *(18)* and Komatsu and Restaino *(17)* who had also shown the benefits of adding novobiocin to salmonellae plating media.

Proteus cultures, like those of salmonellae did not ferment lactose and, as a result, *Proteus* CFUs often look like salmonellae on selective plating media. *Proteus* is a common contaminant of meat products, and, as a result, many CFUs have to be tested from each selective plate before the presence or absence of salmonellae from the meat samples can be assured. In an effort to improve the efficiency of the plating media, we tested a medium similar to the modified lysine agar of Rappold and Boderijk *(21)* which we supplemented with sodium thiosulfate and ferric ammonium citrate (MLIA/USDA) to enhance H_2S production of salmonellae (Experiment 2). With the exception of *S. paratyphi* A which would normally not be found on meat, greater than 95% of all salmonellae produce H_2S, and greater than 98% of all salmonellae decarboxylate lysine *(12)*. Like salmonellae, *Proteus* do not ferment lactose and most are H_2S (+), but no *Proteus* are known to decarboxylate lysine *(12)*; therefore, salmonellae will appear as purple CFUs with black centers on MLIA/USDA while *Proteus* CFUs are yellow. With the exception of a few strains of *Citrobacter freundii*, no other bacteria have been found to mimic salmonellae on MLIA/USDA.

In our study with fresh and cured meats (Table 4), salmonellae were detected from more samples of each product with MLIA/USDA than with BGS or XLD-N. Little difference was found between BGS and XLD-N and occasionally salmonellae were found on one of these plating media and not on the MLIA/USDA. All three plating media perform adequately for differentiating salmonellae from other isolates, and could be recommended as selective plating media. If only two plating media are to be used, BGS could be recommended over XLD-N because there are no H_2S indicators in BGS and any H_2S negative salmonellae missed by MLIA/USDA would be picked up by the BGS. XLD-N uses an H_2S indicator to facilitate detection of salmonellae isolates. Our study indicates that optimal recovery of salmonellae will be when samples are preenriched in lactose broth at 35°C followed by enrichment of samples in TT broth at 43°C for fresh meats or selenite brilliant green broth at 43°C for cured meat samples. When plates of MLIA/USDA or BGS are streaked from these samples, the vast majority of all CFUs on the plates will be salmonellae.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance of Debbie Posey and Debra Waldrop.

REFERENCES

1. Association of Official Analytical Chemists. 1984. Isolation and identification of *Salmonella* species. p. 701-718. *In* Bacteriological Analytical Manual, 6th ed. Association of Official Analytical Chemists, Inc., Arlington, VA.
2. Bailey, J. S., N. A. Cox, and J. E. Thomson. 1981. Efficiency of selenite cystine and TT enrichment broths for the detection of *Salmonella*. J. Appl. Bacteriol. 51:409-414.
3. Cox, N. A., and A. J. Mercuri. 1978. Recovery of salmonellae from broiler carcasses by direct enrichment. J. Food Prot. 41:521-524.
4. Cox, N. A., J. E. Thomson, and J. S. Bailey. 1981. Sampling of broiler carcasses for *Salmonella* with low volume water rinse. Poultry Sci. 60:768-770.
5. Cox, N. A., J. E. Thomson, and J. S. Bailey. 1983. Procedure for isolation and identification of *Salmonella* from poultry carcasses. USDA Agriculture Handbook Number 603.
6. D'Aoust, J. Y. 1977. Effect of storage conditions on the performance of bismuth sulfite agar. J. Clin. Microbiol. 5:122-124.
7. D'Aoust, J. Y., and C. Maishment. 1979. Preenrichment conditions for effective recovery of *Salmonella* in foods and feed ingredients. J. Food Prot. 42:153-157.
8. Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11:1-7.
9. Edwards, P. R., A. C. McWhorter, and M. A. Fife. 1956. The occurrence of bacteria of the Arizona group in man. Can. J. Microbiol. 2:281-287.
10. Fagerberg, D. J., and J. S. Avens. 1976. Enrichment and plating methodology for *Salmonella* detection in food. A review. J. Milk Food Technol. 39:628-646.
11. Farmer, III, J. J., A. C. McWhorter, D. J. Brenner, and G. K. Morris. 1984. The *Salmonella-Arizona* group of Enterobacteriaceae: Nomenclature, classification and reporting. Clin. Microbiol. Newsl. 6:63-66.
12. Farmer, III, J. J., B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Ashbury, C. Riddle, H. G. Wathen-Graden, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner. 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. J. Clin. Microbiol. 21:46-71.
13. Gabis, D. A., and J. H. Silliker. 1974. ICMSF methods studies. II. Comparison of analytical schemes for detection of *Salmonella* in high-moisture foods. Can. J. Microbiol. 20:663-669.
14. Galton, M. M., G. K. Morris, and W. T. Martin. 1968. Salmonellae in foods and feeds. Review of isolation methods and recommended procedures. U.S. Dept. of Health, Education and Welfare/Public Health Service, Communicable Disease Center, Atlanta, Georgia.
15. Greenfield, J., C. H. Bigland, and T. W. Dukes. 1971. The genus *Arizona* with special reference to *Arizona* disease in turkeys. Vet. Bull. 41:605-612.
16. Hajna, A. A., and S. R. Damon. 1956. New enrichment and plating media for the isolation of *Salmonella* and *Shigella* organisms. Appl. Microbiol. 4:341-345.
17. Komatsu, K. K., and L. Restaino. 1981. Determination of the effectiveness of novobiocin added to two agar plating media for the isolation of *Salmonella* from fresh meat products. J. Food Safety 3:183-192.
18. Moats, W. A. 1978. Comparison of four agar plating media with and without added novobiocin for isolation of salmonellae from beef and deboned poultry meat. Appl. Environ. Microbiol. 36:747-751.
19. Mossel, D. A. A., and M. A. Ratto. 1970. Rapid detection of sublethally impaired cells of *Enterobacteriaceae* in dried foods. Appl. Microbiol. 20:273-275.

Reprinted from
Journal of Food Protection, Vol. 47, No. 4, Pages 299-302 (April 1984)

M-9
Buffered Peptone

Recovery of *Salmonella* from Artificially Contaminated Poultry Feeds in Non-Selective and Selective Broth Media

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(Received for publication June 23, 1983)

ABSTRACT

The efficacy of four preenrichment media (lactose broth, lactose broth with Tergitol, buffered peptone and M-9) and of direct enrichment in selenite cystine and modified tetrathionate broths for recovering two *Salmonella* serotypes from dry poultry feed was determined. The salmonellae, artificially inoculated and stored for 7 wk in the dry feed (1.5 to 2.2 cells of salmonellae/g of feed analyzed), were recovered from significantly more samples after preenrichment with M-9 or buffered peptone than with any of the other procedures tested.

Reliable procedures for enumerating *Salmonella* in dry feed samples are needed to accurately determine: (a) the level of naturally occurring *Salmonella* in feed, (b) the level of *Salmonella* in feed required to cause infection in live animals, and (c) process modifications that would eliminate *Salmonella* from feed.

Vegetative bacterial cells exposed to drying may undergo cellular damage and lose their ability to multiply in selective media (*14*). Current isolation procedures used to detect salmonellae in dried foods and feeds generally include a repair step in a non-selective preenrichment medium to recover sublethally injured cells that may be present. Using sublethally heat-stressed *Salmonella typhimurium*, Gomez et al. (*11*) obtained a higher recovery on a minimal agar medium (M-9) than with trypticase soy yeast extract agar. Wilson and Davies (22) have also shown the advantage of preenrichment in using the chemically defined M-9 medium, as compared with complex media, in the repair of heat-injured *Salmonella senftenberg* cells. In contrast, Poelma et al. (*15*) found M-9 to be less efficient than lactose broth for recovery of artificially inoculated salmonellae from casein powder.

The objective of this study was to compare several broth media for the recovery of salmonellae from artificially contaminated poultry feed stored for 7 wk. The performance of direct enrichment (no preenrichment) was also evaluated.

MATERIALS AND METHODS

Test organisms

Two *Salmonella* serotypes (*Salmonella heidelberg* and *Salmonella montevideo*), both resistant to 1,000 mg of nalidixic acid per L, were used as test organisms. Cells were grown for 24 h at 37°C on MacConkey agar slants with 100 mg of nalidixic acid per L added (MCNA), and then washed from the slant with sterile physiological saline. The suspension was diluted with saline to an optical density of 0.2 at 540 nm, and then serially diluted to obtain the desired inoculum level.

Inoculation

Commercial poultry feed (11% moisture and 0.69 a_w) that tested negative for salmonellae was used in these experiments. The feed was inoculated in batches of 1,500 g and mixed in a twin-shell blender as described by Cox et al. (*3*). Addition of 10 ml of wet inoculum increased the moisture content of the feed by less than 0.3%. The average number of cells inoculated (about 10,000 cells/g) was determined by spreading 0.1-ml portions from each dilution onto plates of brain heart infusion agar, then incubating at 37°C for 24 h. Both the level and the uniformity of distribution of the inoculum in the feed were determined by three replicates of a 5-tube most probable number (MPN) procedure using buffered peptone (BP) as the diluent and streaking from the tubes onto plates of MCNA agar. The inoculated feeds were then stored in polyethylene bags for 7 wk at 25°C.

Viable salmonellae were monitored in the stored samples with a 10-tube MPN procedure in which BP was the diluent and samples from tubes were streaked onto MCNA agar plates. MPN determinations were calculated using the tables published by de Man (*6*).

Media

The M-9 medium (*11*) contained Na_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; dextrose, 2.0 g; and distilled water, 1000 ml. All the ingredients except dextrose were dissolved and autoclaved in 90% of the water; dextrose was dissolved in the remaining 10% of the water and autoclaved separately.

Buffered peptone (BP) contained peptone, 10.0 g; NaCl, 5.0 g; Na_2HPO_4, 3.6 g; KH_2PO_4, 1.5 g; and distilled water, 1000 ml (*7*).

All other media were made from commercially prepared dehydrated media (Difco).

Determination of efficiency of preenrichment and enrichment media

After 7 wk of storage, 1-g samples of the two inoculated feed lots were qualitatively analyzed in 35 replicates for each of the two *Salmonella*

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serotypes and each of the six preenrichment/direct enrichment procedures. The preenrichment media evaluated were M-9, BP, lactose broth (LB) and lactose broth with 0.6% Tergitol Anionic 7 (LBT). Selenite cystine (SC) and modified tetrathionate (TT) broths were tested as direct enrichment media. TT broth is the Hajna and Damon (*12*) modification of tetrathionate broth. Media (9 ml/g of feed) were incubated at 37°C for 24 h. Samples of all preenrichment media were streaked onto MCNA agar plates, and samples of direct enrichment were streaked onto MCNA and BG sulfa (BGS) agar plates. The pH of each media plus feed was tested before and after incubation with a Radiometer PHM 63 pH meter (Radiometer A/S, Copenhagen, Denmark). Preenrichment cultures (1-ml portions) were then transferred to 9 ml of SC or TT broth, incubated for 24 h at 37°C (SC and TT) and 43°C (TT), and streaked for isolation onto plates of BGS and MCNA agar plates. All the agar plates were incubated for 24 h at 37°C.

No extraneous organisms grew on MCNA agar; however, on BGS agar the suspected colonies of *Salmonella* had to be selected amidst many other colonies. Three typical colonies were picked from each BGS plate and biochemically and serologically confirmed to be the marker organisms. Their identity was also confirmed on MCNA agar. Colonies that were obviously not *Salmonella* were randomly selected from the BGS plates and identified using the Micro-ID identification system (General Diagnostics, Morris Plains, NJ).

Statistical analysis

The data were statistically analyzed using the categorical data analysis method (Funcat Procedure) in SAS (*16*).

RESULTS AND DISCUSSION

Salmonellae in naturally contaminated dried foods and feeds are usually present in relatively low numbers and are almost always outnumbered by other organisms including other *Enterobacteriaceae* (*21*). In addition, many of the salmonellae cells that survived the drying process and storage in the dry product may have been stressed (*14*).

In the evaluation of procedures for the isolation of salmonellae, either artificially or naturally contaminated feeds are commonly used. The inoculum level should be realistic, and the physiological state of the added organisms should be taken into consideration. Artificial inoculation usually results in a fair distribution of the inoculum in the product. So that subsamples can be compared, and there is no need for large numbers of replicates to make valid comparisons. Naturally contaminated samples usually have an uneven distribution of salmonellae within a batch of feed, the analysis of large numbers of samples is required for a valid comparison.

The inoculation procedure of Cox et al. (*3*) was used in this study to produce feed samples containing well-distributed low numbers of salmonellae. Analysis of the two batches of inoculated feed after the 7-wk storage period found average *Salmonella* counts of 1.5 (*S. heidelberg*) and 2.2 (*S. montevideo*) viable organisms per gram of feed.

The advantage of preenrichment in the isolation of salmonellae from dried material seems to depend on the numbers and the physiological state of the salmonellae present, and the numbers and types of contaminating bacteria present.

The relatively low recovery rates obtained when the feed samples were directly enriched in SC or TT broth (Table 1) suggest that the 7-wk storage period used in this study had produced sublethally injured cells that were recoverable only after incubation in the less inhibitory preenrichment media.

In a study with artificially inoculated poultry feed, Cox et al. (*4*) obtained better results with direct enrichment in SC or TT than with preenrichment in lactose broth followed by enrichment in SC or TT.

Lactose broth is a preenrichment medium widely recommended for the isolation of *Salmonella* from foods and feeds (*1,8,20*). However, recovery rates obtained with LB were much lower than with BP or M-9 (Table 1). This difference could be partly explained by the greater decrease in pH during incubation of the feed samples in LB than during incubation in BP or M-9 (Table 2). This lowering of pH, the extent of which depends on the extraneous flora present, may be detrimental to the repair of injured salmonellae. Wilson and Davies (*22*) found that combination of lactose broth and bone meal was toxic to thermally injured *S. senftenberg*.

The addition of Tergitol 7 to lactose broth has been suggested for products in which the fat content may prevent adequate dispersion of the product (*8,10,19*), and the use of LBT with feeds has been reported by several researchers. Our results suggest that Tergitol 7 does not always improve recovery and may be counterproductive to the recovery of *Salmonella* under certain circumstances (Table 1). Ray et al. (*17*) reported lower recoveries of salmonellae from dried milk products when Tergitol 7 was added to lactose broth.

Preenrichment (except preenrichment of samples with *S. heidelberg* in LB or LBT) yielded more positive recoveries than direct enrichment of the feed samples in SC or TT broth (Tables 1 and 3). Incubation of TT at 37°C usually yielded higher recoveries than did incubation at 43°C, and direct enrichment in SC and in TT broth were similar. It has been previously reported that tetrathionate broth incubated at 43°C was frequently lethal to salmonellae (*13*).

The use of strains resistant to nalidixic acid allowed us to evaluate the performance of preenrichment/enrichment media by plating on MCNA agar, apart from the practical difficulties of isolating *Salmonella* from BGS agar plates in the presence of the other *Enterobacteriaceae*. *Enterobacter agglomerans*, *Enterobacter cloacae* and *Klebsiella pneumoniae* were the predominant *Enterobacteriaceae* isolated from feed samples on BGS agar plates.

Of the preenrichment media, M-9 yielded the highest and BP the second highest recoveries. However, statistical analysis has shown that M-9 is not significantly different from BP. Both M-9 and BP yielded significantly higher recoveries ($P<0.05$) than LB, LBT or direct enrichment with SC or TT. Smith (*18*) demonstrated that as few as 2 to 3 cells of salmonellae per 100 g of animal feed were detectable with a buffered peptone water preenrichment technique. After preenrichment in M-9 or BP, *Salmonella* grew equally well both in SC and TT broth. However, comparison of the results obtained with MCNA (Table 1) with those of BGS agar (Table 3) suggests that SC did not inhibit growth of extraneous organisms sufficiently, thereby making the isolation of *S. heidelberg* difficult.

The performance of various preenrichment and enrichment media in the recovery of salmonellae from dry feeds and feed ingredients has been compared in many studies (*2,4,5,9*) which have reported a variety of results and conclusions. Thus, the unbiased reader will have difficulty in deter-

TABLE 1. *Recovery of* Salmonella *from artificially inoculated dry feed with different preenrichment and enrichment media and growth on plates of MCNA agar.*

Organism	Preenrichment	% of *Salmonella* positive samples[a]				
			Enrichment medium			
			SC 37°C	TT 37°C	TT 43°C	Mean[b]
S. heidelberg	LB	20	20	23	6	17 B
	LBT	14	14	14	14	14 B
	BP	51	51	51	46	50 A
	M-9	60	57	60	54	58 A
	None[c]	-	20	14	NT[d]	
S. montevideo	LB	63	60	63	46	57 C
	LBT	69	69	69	66	68 C
	BP	83	83	83	80	82 B
	M-9	89	89	89	83	87 A
	None	-	46	57	NT	
Total	LB	41	40	43	26	37 B
	LBT	41	41	41	40	41 B
	BP	67	67	67	63	66 A
	M-9	74	73	74	69	72 A
	None	-	33	36	NT	

[a]Each of the preenrichment or direct enrichment media was tested with 35 feed samples containing one serotype. Data presented were calculated from the number of *Salmonella*-positive samples out of 35 for each serotype or out of a total of 70 samples for the two serotypes.
[b]Means within a row followed by the same letter were not significantly diferent (P = 0.05).
[c]None, direct enrichment in SC or TT broth.
[d]NT, not tested.

TABLE 2. *Changes in pH in various preenrichment media after the addition of feed samples inoculated with* S. heidelberg.

Preenrichment medium	pH of medium	pH after addition of feed	
		Before incubation	After 24 h incubation
LB	6.8	6.8	4.8-5.5
LBT	7.0	6.8	5.5-5.8
BP	7.1	6.9	5.8-6.4
M-9	7.0	6.9	5.9-6.2

mining the most suitable procedure. One important reason for this problem is that media usually have been selected on an empirical basis, whereas monitoring of factors, which may explain the results obtained, is limited in most of those studies. Factors that are likely to influence the results obtained with different preenrichment/enrichment procedures are: (a) choice of medium; (b) incubation temperature; (c) the numbers of salmonellae present in the product and their physiological state; (d) the types and numbers of other bacterial contaminants able to multiply in the medium and their ability to overgrow the salmonellae; and (e) the ability of bacterial contaminants present to cause changes in the medium that affect the growth and survival of salmonellae. Our results indicate that lactose broth should not be used as a preenrichment medium for recovery of salmonellae from feed. A buffered peptone, minimal medium or some similar medium should be used as a preenrichment or recovery medium for feed analysis.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Debbie Posey and Mike Ban, and the clerical assistance of Joy Cornelius.

Mention of specific brand names does not imply endorsement by the authors or institutions at which they are employed to the exclusion of others not mentioned.

REFERENCES

1. AOAC. 1980. Official methods of analyses, 13th ed. Association of Official Analytical Chemists, Washington, DC.
2. Carlson, V. L., and G. H. Snoeyenbos. 1974. Comparative efficacies of selenite and tetrathionate enrichment broths for the isolation of *Salmonella* serotypes. Am. J. Vet. Res. 35:711-718.
3. Cox, N. A., J. S. Bailey, and J. E. Thomson. 1982. Evaluation of procedures to disperse inoculated *Salmonella* in poultry feeds. Poultry Sci. 61:382-384.
4. Cox, N. A., J. S. Bailey, and J. E. Thomson. 1982. Effect of various media and incubation conditions on recovery of inoculated *Salmonella* from poultry feed. Poultry Sci. 61:1314-1321.
5. D'Aoust, J. Y., and C. Maishment. 1979. Preenrichment conditions for effective recovery of *Salmonella* in foods and feed ingredients. J. Food Prot. 42:153-157.
6. de Man, J. C. 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. 1:67-78.
7. Edel, W., and E. H. Kampelmacher. 1973. Comparative studies on the isolation of "sublethally injured" salmonellae in nine European laboratories. Bull. W. H. O. 48:167-174.
8. Food and Drug Administration. 1978. Bacteriological analytical manual, 5th ed. Association of Official Analytical Chemists, Washington, DC.
9. Gabis, D. A., and J. H. Silliker. 1974. ICMSF methods studies. VI. The influence of selective enrichment media and incubation tempera-

TABLE 3. *Recovery of* Salmonella *from artificially inoculated dry feed with different preenrichment and enrichment media and growth on BGS agar*[a].

		% of *Salmonella* positive samples[b]			
	Preenrichment	Enrichment media			
		SC 37°C	TT 37°C	TT 43°C	Mean[c]
S. heidelberg	LB	6	9	6	7 B
	LBT	14	9	11	11 B
	BP	26	40	37	34 A
	M-9	34	43	34	37 A
	None[d]	9	11	NT[e]	
S. montevideo	LB	40	14	17	24 B
	LBT	29	54	29	37 B
	BP	66	57	46	56 A
	M-9	74	51	31	52 A
	None	11	9	NT	
Total	LB	23	11	11	15 B
	LBT	21	31	20	24 B
	BP	46	49	41	45 A
	M-9	54	47	33	45 A
	None	10	10	NT	

[a]Suspected colonies picked from BGS agar plates and further confirmed as the marker organism.
[b]See Table 1 footnote "a" for explanations.
[c]Means within a row followed by the same letter were not significantly different (P = 0.05).
[d]None, direct enrichment in SC or TT broth.
[e]NT, not tested.

tures on the detection of *Salmonella* in dried foods and feeds. Can. J. Microbiol. 20:1509-1511.

10. Galton, M. M., G. K. Morris, and W. T. Martin. 1968. Salmonellae in foods and feeds. Review of isolation methods and recommended procedures. Public Health Service Publication, National Communicable Disease Center, Atlanta, GA.
11. Gomez, R. F., A. J. Sinskey, R. Davies, and T. P. Labuza. 1973. Minimal medium recovery of heated *Salmonella typhimurium* LT 2. J. Gen. Microbiol. 74:267-274.
12. Hajna, A. A., and S. R. Damon. 1956. New enrichment and plating media for the isolation of *Salmonella* and *Shigella* organisms. Appl. Microbiol. 4:341-345.
13. McCoy, J. H. 1962. The isolation of salmonellae. J. Appl. Bacteriol. 25:213-224.
14. Ordal, Z. J., J. J. Iandola, B. Ray, and A. G. Sinskey. 1976. Detection and enumeration of injured microorganisms. pp. 163-169. *In* M. L. Speck (ed.) Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, DC.
15. Poelma, P. L., W. H. Andrews, and C. R. Wilson. 1981. Comparison of methods for the isolation of *Salmonella* species from lactic casein. J. Food Sci. 46:804-809.
16. Ray, A. A. 1982. SAS user's guide: statistics. SAS Institute, Cary, NC. pp. 257-285.
17. Ray, B., J. J. Jezeski, and F. F. Busta. 1972. Isolation of salmonellae from naturally contaminated dried milk products. J. Milk Food Technol. 35:607-614.
18. Smith, P. J. 1977. A standard technique for the isolation of *Salmonella* from animal feeds. J. Hyg. 79:449-461.
19. U. S. Department of Agriculture. 1968. Recommended procedures for the isolation of *Salmonella* organisms from animal feeds and feed ingredients. ARS 91-68. Animal Health Division, Agricultural Research Service, Hyattsville, MD.
20. U. S. Department of Agriculture. 1974. Microbiology laboratory guidebook. Food Safety and Quality Service, Scientific Services, Washington, DC.
21. van Schothorst, M., and E. H. Kampelmacher. 1968. Comparative studies on the isolation of salmonellae from dry feed ingredients. Proc. 6th Intl. Symp. Food Microbiol., Bilthoven, Netherlands. pp. 193-209.
22. Wilson, J. M., and R. Davies. 1976. Minimal medium recovery of thermally injured *Salmonella senftenberg* Y969. J. Appl. Bacteriol. 40:365-374.

Reprinted from
Journal of Food Protection, Vol. 46, No. 9, Pages 764-766 (September 1983)

Micro - ID
Serology

Rapid Procedure for Biochemical Characterization and Serological Confirmation of Suspect *Salmonella* Isolates

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(Received for publication November 1, 1982)

ABSTRACT

Fifty-two freshly processed broiler carcasses were examined for the presence of *Salmonella* by using a rinse method. Three selective plating media (bismuth sulfite, brilliant green sulfa and Hektoen enteric)were compared. After 24 h of incubation, typical colonies were picked from each selective plate. An 8-h procedure to biochemically characterize (Micro ID) and serologically (poly O and poly H) confirm *Salmonella* was then compared with a conventional procedure. Suspect *Salmonella* isolates were correctly classified from 63% of the carcasses with both the 8-h and conventional procedures. Of the 244 isolates confirmed to be *Salmonella* by conventional testing, 236 (97%) were also confirmed by the 8-h procedure. Brilliant green sulfa and Hektoen enteric agar were superior to bismuth sulfite agar for *Salmonella* recovery. The 8-h procedure required less incubation time (8 h vs. 48 h) after colony formation, less incubation space, and less media preparation and cleanup than the conventional procedure.

Conventional procedures for examining foods and feeds for salmonellae involve preenrichment with nonselective media when the organisms in the sample are stressed, enrichment in selective media, selective plating to obtain isolated colonies, biochemical screening with triple sugar iron (TSI) and lysine iron (LI) agar slants, further biochemical testing and serological confirmation with somatic poly O and flagellar poly H antisera. These are very time-consuming procedures, i.e., 72 h are required for colonies to form (48 h if preenrichment is not used) and an additional 48 to 96 h for biochemical characterization and serological confirmation. Cox and Mercuri (*1*) proposed an alternative procedure requiring only 24 h from colony formation by concurrently doing biochemical tests and serology with the Minitek system (BBL).

With increased public awareness of food poisoning bacteria and possible increased scrutiny by regulatory agencies, it is particularly important to develop methods for rapid and accurate *Salmonella* detection. An enrichment serology (ES) procedure to detect *Salmonella* in dried foods and feeds using only broth cultures and serological reactions was proposed by Sperber and Deibel (*10*). Mohr et al. (*8*) used a 6-h ES procedure to screen for *Salmonella* from a variety of food products, and Surdy and Haas (*11*) used a 6-h modified ES procedure to detect *Salmonella* in dried soy products. While these procedures are very rapid, they alone, without biochemical characterization to complement serology, could result in false-negatives which are impossible to detect. The results of ES are highly correlated (>95%) with those of conventional procedures for recovering *Salmonella*, but they have not become widely accepted because of the reluctance of many microbiologists to forego biochemical characterization.

Silliker et al. (*9*) described a procedure for serologically confirming *Salmonella* from isolated colonies on selective plating media. This method is rapid and accurate, but since a battery of biochemical tests are not run, they recommend simultaneously inoculating TSI/LI slants, which requires an additional 24 h of incubation. A 4-h system (Micro-ID) was found to be accurate for the biochemical characterization of *Salmonella* isolates (*2,3*). Therefore, the objective of this study was to evaluate rapid biochemical (Micro-ID) and concurrent serological tests to confirm suspect-*Salmonella* colonies in 8 h, and to compare the Micro-ID serology procedure to the time-consuming conventional procedure.

MATERIALS AND METHODS

A total of 52 freshly processed broiler carcasses were rinse-sampled according to the procedure of Cox et al. (*4*). The rinse samples were incubated for 16 h at 37°C and then brilliant green sulfa (BGS)[1], Hektoen enteric (HE) and bismuth sulfite (BiS) agar plates were streaked for isolation. After 24 h of incubation at 37°C, up to three suspect *Salmonella* colonies per plate were picked and inoculated onto triple sugar iron (TSI) and lysine iron (LI) agar slants for the conventional confirmation procedure, and into tubes containing 0.7 ml of M broth (*10*) to run the modified 8-h confirmation procedure (Table 1).

[1]*All media were from the Difco Company; this does not imply endorsement by the authors or organization by which they are employed to the exclusion of others not mentioned.*

TABLE 1. *Confirmation procedure (8-h) for suspect-*Salmonella *isolates.*

1. Pick typical colony to 0.7 ml M broth.
2. Incubate M broth at 37°C for 6 h.
3. After 4 h, remove 2 loopfuls of M broth and run slide poly O agglutination.
4. After 4 h, if poly O (+), remove 0.2 ml M broth, add to 3.2 ml sterile saline and run Micro-ID.
5. After 6 h of incubation, run tube poly H agglutination on remaining 0.5 ml M broth and incubate for 2 h in a 50°C water bath.
6. After 8 h, read Micro-ID and poly H agglutination results.

In the conventional confirmation procedure, TSI/LI slants were incubated at 37°C for 24 h. Brain heart infusion (BHI) plates were streaked from TSI/LI agar slants exhibiting a typical *Salmonella* reaction to determine purity and to produce isolated colonies for agglutination tests. Standard poly O slide and poly H tube agglutination tests (*6*) were then made.

In the 8-h confirmation procedure (Table 1), biochemical tests and serology (both O and H) were performed concurrently. Biochemical confirmation was determined by using the Micro-ID system (General Diagnostics, Division of Warner-Lambert Company, Morris Plains, New Jersey).

TABLE 2. *Comparison of an 8-h procedure with a conventional procedure for the detection of* Salmonella *on broiler carcasses.*

Procedure	Carcasses	Isolates
Conventional	33/52[a]	244/307[b]
8-h	33/52	236/307

[a]Number of *Salmonella*-positive carcasses/number of carcasses sampled.

[b]Number of isolates confirmed to be *Salmonella*/number of typical isolates selected.

RESULTS AND DISCUSSION

The conventional and the 8-h confirmation procedures both showed positive recoveries of *Salmonella* from 33 of 52 (63%) of the carcasses sampled (Table 2). Of 244 isolates that were confirmed to be *Salmonella* by the conventional procedures, 236 (97%) were also confirmed by the 8-h procedure. Of the eight cultures identified by the conventional procedure but not by the 8-h system, seven were incorrectly identified because of an initially false-negative poly H agglutination test. However, because the isolates were biochemically confirmed as *Salmonella* by the Micro ID system, the poly H test was rerun and the cultures were correctly identified the second time. Only one culture was not properly identified as *Salmonella* by the Micro ID system.

The effectiveness of three commonly used selective plating media was compared with isolates from the conventional testing procedure. BGS and HE recovered *Salmonella* from 33 (63%) and 32 (62%) carcasses, respectively, whereas BiS only recovered *Salmonella* from 24 (46%) carcasses. Although BiS was not as efficient for recovery of *Salmonella* as the other two media in this study, its use is recommended because BiS is the only *Salmonella* plating medium not based on the fermentation of lactose and, therefore, is the only medium that would detect the 1% of salmonellae which are lactose-positive (*7*).

TABLE 3. *Percentage of isolates from selective enrichment media that were confirmed to be* Salmonella.

Selective plating medium used	Typical isolates selected	Isolates confirmed to be *Salmonella*
BiS	72	60 (83%)
BGS	100	95 (95%)
HE	135	89 (66%)
Total	307	244 (79%)

The percentage of confirmations, using conventional procedures, of typical, *Salmonella*-like colonies selected from the various plating media are shown in Table 3. BGS was the most efficient; 95% of the colonies tested were confirmed as *Salmonella*. Colonies of *Proteus* and a few other H_2S-positive *Enterobacteriaceae* appeared similar to those of *Salmonella* on BiS and HE (*5*).

The advantages of the 8-h confirmation procedure over conventional procedures were: (a) 24 to 48 h less time between sampling and confirmation and (b) less time required for media preparation, inoculation and cleanup. The advantage of the 8-h procedure over the ES procedure was that both serological confirmation and biochemical characterization were obtained in 8 h. In the 8-h system, however, more isolates must be tested than in the conventional procedure because the 8-h procedure has no TSI/LI screening step, and 24 h longer were required than with ES.

Many factors must be considered when determining which procedure is best for detection of the presence of *Salmonella* in a food product. If rapid results are not required, then conventional procedures might be preferred; however, if release of a food product must be delayed until freedom from *Salmonella* contamination is assured, the 8-h procedure would be desirable.

In conclusion, the 8-h confirmation procedure agreed with the conventional procedure for 97% of the isolates tested. Also, all carcasses that were found to have *Salmonella* by conventional procedures also showed *Salmonella* by the 8-h procedure.

The time necessary for determining if a colony is *Salmonella* or not has been reduced by running the biochemical, poly O and poly H confirmation steps concurrently, rather than consecutively. Further study is underway to reduce the time from sampling of the food product to final confirmation of *Salmonella* to less than 24 h without sacrificing accuracy.

ACKNOWLEDGMENTS

Special thanks are extended to Ms. Debbie Posey and Mr. Mike Ban for their excellent technical assistance, and to Ms. Joy Cornelius for her very able clerical assistance.

REFERENCES

1. Cox, N. A., and A. J. Mercuri. 1976. Rapid confirmation of suspect *Salmonella* colonies by use of the Minitek system in conjunction with serological tests. J. Appl. Bacteriol. 41:389-394.

2. Cox, N. A., and A. J. Mercuri. 1979. Accuracy of Micro-ID for identification of *Salmonella* and other *Enterobacteriaceae* from chemical and food sources. J. Food Prot. 42:942-945.
3. Cox, N. A., J. S. Bailey, and J. E. Thomson. 1981. Comparison of Micro-ID and Minitek-serology systems for rapid identification of *Salmonella*. J. Food Prot. 44:442-444.
4. Cox, N. A., J. E. Thomson, and J. S. Bailey. 1981. Sampling of broiler carcasses for *Salmonella* with low volume water rinse. Poultry Sci. 60:768-770.
5. Difco Laboratories. 1953. Difco manual of dehydrated culture media and reagents for microbiological and chemical laboratory procedures. Difco Laboratories, Detroit, MI.
6. Edwards, P. R., and W. H. Ewing. 1972. Identification of *Enterobacteriaceae*, 3rd ed. Burgess Publishing Company, Minneapolis.
7. Ewing, W. H. 1973. Biochemical reactions given by *Enterobacteriaceae* in commonly used tests. United States Department of Health Education and Welfare, Publication Number (CDC) 74-8269, Washington, DC.
8. Mohr, H. K., H. L. Trenk, and M. Yeterian. 1974. Comparison of fluorescent-antibody methods and enrichment serology for the detection of *Salmonella*. Appl. Microbiol. 27:324-328.
9. Silliker, J. H., P. T. Fagan, J. Y. Chiu, and A. Williams. 1965. Polyvalent H agglutination as a rapid means of screening non-lactose-fermenting colonies for *Salmonella* organisms. Am. J. Clin. Pathol. 43:548-554.
10. Sperber, W. H., and R. H. Deibel. 1969. Accelerated procedure for *Salmonella* detection in dried foods and feeds involving only broth cultures and serological reactions. Appl. Microbiol. 17:533-539.
11. Surdy, T. E., and S. O. Haas. 1981. Modified enrichment-serology procedure for detection of salmonellae in soy products. Appl. Environ. Microbiol. 42:704-707.

Reprinted from
Journal of Food Protection, Vol. 45, No. 14, Pages 1332-1337 (December 1982)

Exhibit 4a #41

Methods for Recovery of *Campylobacter jejuni* from Foods

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(Received for publication June 4, 1982)

ABSTRACT

The triangular relationship between *Campylobacter jejuni*, foods and disease in humans has been well-documented. Many studies have revealed that *C. jejuni* causes at least as many cases of human gastroenteritis as does *Salmonella* sp. Foods are an important vehicle in human infection, and raw milk is most frequently implicated. Other animal products also serve as potential sources of infection. *C. jejuni* has been found on the carcasses of poultry and other domestic animals throughout the world. The organism is microaerophilic and various methods for establishing appropriate growth conditions, such as the Fortner principle, atmosphere replacement and adding of supplements to encourage growth of *C. jejuni*, are available. Methods developed for use in clinical laboratories lack the necessary sensitivity and selectivity, and therefore have limited use in detecting small numbers of *C. jejuni* in foods. In one enrichment method for detecting *C. jejuni* in foods, washings are filtered and centrifuged, the sediment is suspended in the enrichment broth and the suspension is incubated under a constant gas flow at reduced oxygen levels. Following incubation enrichment broth is filtered and plated onto selective media. In another recently developed method, food samples are directly added to an enrichment broth with antibiotics and incubated under a microaerobic atmosphere before selective plating. Butzler's, Skirrow's and Campy-BAP selective media use several antibiotics to which *C. jejuni* is resistant. The plates are supplemented with horse or sheep blood, depending upon the specific formulation. The optimum temperature for growth of *C. jejuni*, about 42°C, may also be used for selection. It is now possible to recover 0.1 to 1 cell of *C. jejuni* per 10 to 25 g of food sample from among 10^6 to 10^9 indigenous bacteria. After a characteristic colony is isolated, the key criteria for presumptive identification of *C. jejuni* by phase-contrast microscopy are darting, corkscrew motion and a comma to spiral shape.

The bacterium *Campylobacter jejuni*[1] has only recently achieved recognition as an important pathogen of gastroenteritis in humans. *C. jejuni* is recovered from human diarrheal specimens at a rate that depends on both the awareness of the investigators and the methods employed. This is borne out by following the increase in number of cases of *Campylobacter* enteritis reported to the communicable Disease surveillance Centre in Great Britian (*1*) (Fig. 1). Observations confirming *C. jejuni* as an important agent of gastroenteritis have been well-documented. In one study involving diarrhetic children from Montreal, *C. jejuni* was isolated from fecal specimens at a rate of 4.3%, *Salmonella* at 5.1%, *Yersinia enterocolitica at 2.8% and Shigella* at 1.4% (*30*). In Sweden, when stool samples from about 2,550 patients with gastrointestinal infections were cultured for bacterial pathogens, *Campylobacter* was recovered from 277 samples and *Salmonella* from 183 (*48*). As the true incidence of *Campylobacter* infection of humans is becoming understood, the general assessment is that the pathogen is at least as prevalent as *Salmonella* in patients with gastroenteritis.

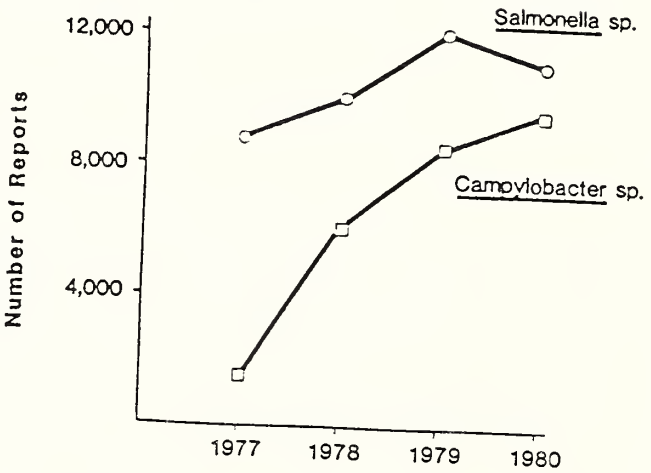

Figure 1. *Annual reports of gastrointestinal pathogens over a four-year period. With increasing awareness of* Campylobacter *sp., the number of reports approach those attributed to* Salmonella *sp.* (1).

[1]*Note that the following names have been used in the past for this organism and represent a close estimation of the same bacteria:* C. jejuni/coli (54), C. fetus *ssp.* jejuni (41), *"related" vibrios* (21), *and* Vibrio fetus (44). *A detailed review of the taxonomy and nomenclature for this organism is presented by Doyle* (10) *in his review of the genus. The name* C. jejuni *will be used in this paper.*

FOODBORNE GASTROENTERITIS

The increased awareness of the presence of *C. jejuni* in patients with gastroenteritis has lead to attempts to recover the pathogen from incriminated foods. The organism so far has been associated with pork, ground beef, chicken and milk. Unpasteurized milk is the most frequently implicated vehicle of *Campylobacter* infection. *Campylobacter* enteritis was first suspected as being attributed to the consumption of milk in 1946 (*24*). At two institutions with the same milk supplier, a large outbreak involved 151 individuals. The milk was thought to be the vehicle for two reasons: (a) raw milk could have been shipped by mistake and (b) microscopic examination of fecal smears from 31 patients yielded almost pure cultures of a vibrio-like microorganism that conformed to characteristics of *C. jejuni*. In addition to this outbreak, numerous other reports have documented the association of *Campylobacter* enteritis with unpasteurized milk (*3,34,36,50-52*). This list is not exhaustive and, indeed, Butzler and Skirrow (*7*) mention that consumption of milk was implicated in five major *Campylobacter* outbreaks in Great Britain during a 6-month period.

Consumption of cake, particularly the icing, was associated with an outbreak of *Campylobacter* enteritis as reported by Blaser et al. (*4*). In a report from the Netherlands (*5*), an explosive outbreak of *Campylobacter* enteritis occurred among soldiers on a survival exercise. Of 123 cadets given live chickens to prepare for their evening meals, 89 became ill with symptoms of enteritis within the following week. Fecal samples from 104 of the cadets yielded no *Salmonella* or *Shigella*, but 34 samples yielded *Campylobacter*. The authors speculated that improper heating of the chickens left viable pathogens in the food. Raw hamburger has also been implicated as a source of *Campylobacter* enteritis in a military camp (*28*). Other reports further implicate *C. jejuni* as the causative agent in foodborne enteritis, and only very small numbers (500 cells) are needed to effect a gastroenteritic response in humans (*35*).

ASSOCIATION WITH FOODS

Smith and Muldoon (*43*) were the first to report the incidence of *C. jejuni* from commercially processed poultry. Using comparatively rudimentary methods, they recovered three isolates from 165 poultry meat samples purchased from local retail stores. Subsequently, Simmons and Gibbs reported recovery rates of 48% for processed chickens and 92% for turkeys (*38*). Studies from New York (*13*), Denver (*25*), Sweden (*27*) and Ohio and Ontario (*32*) have reported incidences of 22 to 92% of *C. jejuni* on retail market poultry meat. Soaking turkey carcasses overnight in 340 ppm chlorine wash water did not decrease the number of positive carcasses (*25*).

Like *Salmonella*, *C. jejuni* may also be isolated from red meats, although recovery rates are lower than those for poultry meats. *C. jejuni* has been isolated from lamb carcasses (*45*) and from eviscerated pork, lamb and beef carcasses (*46*). Using direct plating onto a selective medium, Stern recovered *Campylobacter* from unwashed carcasses of pig, lamb and beef at rates of 38, 24 and 2%, respectively. Stern suggested that the same abuses in handling and preparation of meats that result in meatborne outbreaks of salmonellosis may also account for *Campylobacter* infections. Hudson and Roberts (*17*) did not find *C. jejuni* on beef or lamb carcasses but did find it on 59% of the pig carcasses they examined. Turnbull and Rose (*53*) reported that 1.6% of meat samples from both abattoirs and retail outlets were positive for *Campylobacter*. Investigations by Kaijser and Svedhem (*19*) indicate that domestic animals are the most probable source of *Campylobacter* involved in human gastroenteritis, and that the organism is capable of surviving for several days in dairy or meat products stored at 4°C. With these reports on the consistent presence of *Campylobacter* in foods has come the recognition that the same measures employed for prevention of salmonellosis should also be applied to control gastroenteritis caused by campylobacters.

ISOLATION METHODS

Microaerobic requirements

C. jejuni is a strict microaerophile (*42*), and isolation methods must attend to this requirement. Various methods have been used to create appropriate conditions for growth. Karmali and Fleming (*20*) developed a method in which the Fortner principle is used to isolate *Campylobacter* from stools. A rapidly growing *Proteus* sp. is streaked onto half of a blood agar plate to reduce the oxygen tension in a closed system, thus making growth of *Campylobacter* possible on the other half. A method for obtaining a closed environment containing 5% oxygen is described in the *Anaerobe Laboratory Manual* (*16*). Luechtefeld et al. (*26*) found that isolation rates of *Campylobacter* grown in a closed container with 5% oxygen were superior to those grown in a candle jar. In another study comparing the CampyPak II gas generator systems and 5% oxygen in a closed container, no difference in isolation rates of *C. jejuni* was obtained (*6*). A method we have used for the past two years is exchanging the atmosphere of the container used to culture the organism with 5% O_2:10% CO_2:85% N_2. Blood agar plates containing the samples are inverted and placed in a modified anaerobe jar, the air is evacuated with a standard laboratory vacuum line, and the gas mixture is introduced into the jar until a positive pressure is detected coming out of the jar. The jar is sealed with a clamp and placed into an appropriate incubator.

A supplement that improves the aerotolerance of *Campylobacter* has been described (*12,14,15*). It was found that specific quantities of ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP) added to broth and agar increased the oxygen tolerance of *C. jejuni*. It was concluded that these supplementary compounds enhance oxygen tolerance by quenching superoxide anions and hydrogen peroxide that occur spontaneously in the culture medium. FBP has been used to promote growth, and such a suppiement may also be useful in an enrichment broth.

TABLE 1. *Summary of enrichment procedures used to select for* Campylobacter jejuni.

Authors	Reference	Selective agents (per liter)	Sensitivity
Blaser et al.	*2*	Vancomycin, 10 mg Trimethoprim, 5 mg Polymyxin B, 2,500 IU Amphotericin, 2 mg	33% greater than direct plating
Chan and Mackenzie	*8*	a) Same as ref. 2 with 30 mg cephalothin or b) Same as ref. 2, with 50,000 IU polymyxin B	6% greater than direct plating
Tanner and Bullin	*49*	Alkaline peptone water (pH 8.4) under reduced atmosphere	Allowed for recovery of 1-10 organisms
Lander and Gill	*22*	Vancomycin, 20 mg Trimethoprim, 5 mg Polymyxin B, 10,000 IU Actidione, 100 mg 5-Fluorouracil, 500 mg	Not given
Park and Stankiewicz	*31*	Vancomycin, 8 mg Trimethoprim, 4 mg Cephalothin, 3 mg Colistin, 3 mg 1-2 d under constant flow of reduced atmosphere Filtration through filter with 0.65 μm pores	1 cell per 10 g among 10^5 to 10^7 bacteria/g
Doyle and Roman	*11*	Vancomycin, 15 mg Trimethoprim, 5 mg Polymyxin B, 20,000 IU Cyclohexamide, 50 mg Reduced atmosphere for 18 h	1 cell per 10 g among 10^6 to 10^9 bacteria/g

Enrichment methods

Several enrichment methods that may be used to select for *C. jejuni* from specimens containing other indigenous flora are described in Table 1. However, most of these methods were developed in clinical laboratories and do not have the sensitivity and selectivity needed for detection of *C. jejuni* in foods. Although fecal specimens from infected individuals usually contain comparatively large numbers of *Campylobacter* (about 10^6 and 10^8/g), these large numbers may not occur in foods. Only 500 cells in a cup of milk have caused an enteritic response in a human (*35*). Direct plating of swabs from inoculated meat has shown that the organism can be recovered from an indigenous flora of about 10^4 cells/cm^2 when present at levels of at least 32 per cm^2 (*45*). The enrichment methods described below are still in need of comparative studies for sensitivity, selectivity and other practical considerations, such as time, cost and simplicity.

Resistance of *C. jejuni* to several antibiotics is frequently employed in selective enrichment. The organism is most often resistant to vancomycin, which is active against gram-positive cocci. *C. jejuni* is resistant to polymyxin B, trimethoprim lactate and the cephalosporins. Polymyxin B is inhibitory to *Enterobacteriaceae* and *Pseudomonas* sp. while trimethoprim acts against *Proteus* sp. The cephalosporins are active against *Streptococcus faecalis*, *Enterobacter* sp., *Serratia* sp., *Pseudomonas aeruginosa*, some *Proteus* sp., *Y. enterocolitica* and *Bacteroides fragilis*. Amphotericin B and cycloheximide (actidione) are used to inhibit yeasts and fungi.

Blaser et al. (2) described a Campy-thio enrichment broth (thioglycollate broth plus 0.16% agar and 10 mg of vancomycin, 5 mg of trimethoprim, 2500 IU of polymyxin B and 2 mg of amphotericin B per liter) that allowed for 33% greater recovery of *C. jejuni* than did the use of selective medium alone. Stool specimens are inoculated into Campy-thio and held in the refrigerator for 8 h before plating onto selective agar medium. During this enrichment, the low temperature inhibits the growth of *Campylobacter* but the indigenous bacteria are diminished through the action of the antimicrobials.

A similar enrichment procedure, described by Rosef (*37*), enhanced the recovery of *Campylobacter* from the gallblad-

der of pigs. The same concentrations of antimicrobials described by Blaser et al. (*2*) were used, but amphotericin B was omitted. In place of thioglycollate broth and agar, 10 g of peptone, 8 g of "Lab lemco" powder (Oxoid L 29), 1 g of yeast extract, 5 g of NaCl and 16 ml of 0.025% rezasurin were used per liter. The gallbladder sample was added to 100 ml of this enrichment medium, and the samples were incubated at 42°C in a microaerobic atmosphere. The enrichment medium was plated after 24 and 48 h of incubation. In this study *C. jejuni* was isolated only when enrichment medium was used. Forty-two and 58% of the gallbladders sampled were positive for *C. jejuni* after 24 and 48 h of enrichment, respectively.

Chan and Mackenzie recently described two enrichment media used to isolate *Campylobacter* from stools (*8*). The media contained the same antimicrobials as described by Blaser and co-workers (*2*) except with (a) 30 mg of cephalothin added per liter or (b) the polymyxin B concentration was increased to 50 IU/ml. A fecal swab was plunged into a small vial containing the enrichment medium, and the sample was incubated overnight at 42°C under a normal atmosphere. The swab was subcultured onto a selective plate. Using this method, the isolation rate of *C. jejuni* was increased by 6% over direct plating.

Tanner and Bullin (*49*) used alkaline peptone water (pH 8.4) as an enrichment broth. They incubated samples at 43°C in a reduced atmosphere of 5% O_2:10% CO_2:85% N_2. Tanner and Bullin found that this procedure enabled them to recover as few as 1 to 10 cells of *Campylobacter* and allowed the organisms to proliferate in the presence of large numbers of *Escherichia coli* and *S. faecalis*. With this broth, fecal samples that were negative by direct plating methods were positive for *Campylobacter*.

Lander and Gill (*22*) used a combination of enrichment and direct plating to determine whether *C. jejuni* could infect a bovine udder. The selective components of their enrichment broth consisted of 40 mg of vancomycin, 20 mg of trimethoprim, 10,000 IU of polymyxin B, 100 mg of actidione and 500 mg of 5-fluorouracil per liter. Fecal samples enriched in 20 ml of this broth at 37°C for 2 d in air were subcultured onto selective agar media. In a similar enrichment medium, which also contained 1.5% ox bile, Oosterom et al. (*29*) increased recovery sensitivity, enabling detection of 3 to 10 cells of *C. jejuni* per g of meat.

In an article on the prevalence of *C. jejuni* in fresh eviscerated whole market chickens, Park et al. (*32*) described a method that included an enrichment step before selective plating. Store bought chickens were washed in 250 ml of nutrient broth, and the broth was filtered through cheesecloth. The filtrate was centrifuged and the bacteria in the sediment were resuspended in 5 ml of brucella broth. After part of the suspension was streaked directly onto selective agar media, the remainder was transferred to 100 ml of enrichment broth. The enrichment broth contained 8 mg of vancomycin, 4 mg of trimethoprim, 3 mg of cephalothin, 3 mg of colistin and 30 ml of calf serum per liter of brucella broth. Since the original publication, this broth has been modified to exclude TRIS buffer, thereby reducing the pH to 7.2 (Park, personal communication). Flasks containing the suspended sample and enrichment broth were incubated at 42°C for 1 to 2 d under a constant flow of 5 to 7 ml of 5% O_2:10% CO_2:85% N_2 per minute. After incubation, 5 ml of enrichment broth were filtered through a 0.65-μm membrane filter, and the filtrate was plated onto two selective media. This enrichment system was capable of recovering 1 cell of *C. jejuni* per 10 g in the presence of 10^4 to 10^6 indigenous organisms per gram of chicken (*31*). Direct plating procedures yielded a 32% recovery rate, whereas the enrichment method of Park et al. yielded a 62% recovery rate for *C. jejuni* from store bought chickens (*32*).

Most recently an enrichment technique has been reported that is capable of recovering 0.1 to 1 cell of *C. jejuni* per g of food containing as many as 10^6 to 10^9 indigenous bacteria (*11*). The effectiveness of this method has been confirmed by others. Ten or 25 g of food are suspended in 90 or 100 ml, respectively, of an enrichment broth consisting of 7% lysed horse blood, 3 g of sodium succinate, 0.1 g of cysteine hydrochloride, 15 mg of vancomycin, 5 mg of trimethoprim, 20,000 IU of polymyxin B and 50 mg of cycloheximide per liter of brucella broth. The inoculated broth is held in a flask, and the atmosphere is evacuated and replaced three times with a gas mixture of 5% O_2:10% CO_2:85% N_2. The flask is incubated at 42°C for 16 to 18 h at 100 gyrations per minute. After incubation, the enrichment broth is plated onto selective agar plates, and these plates are incubated at 42°C for 48 h under microeaerobic conditions. This method is sensitive, comparatively simple and requires less time to recover *C. jejuni* from foods than other reported methods.

Selective plating

Three selective agar media have emerged as most popular among the plating media developed for isolation of *C. jejuni* from stools of patients with gastroenteritis. What is known as the "Butzler" formulation evolved from the original work of Dekeyser et al. (*9*) in Butzler's laboratory. Their isolation technique involved suspending stools in broth, allowing the suspension to settle for 1 h and centrifuging the resultant supernatant fluid at 1,500 × *g* for 5 min. Four milliliters of the resulting supernatant fluid were filtered through a 0.65-μm Millipore filter. The final 0.3 ml of this filtrate was surface-plated onto selective agar. Their selective medium contained 15% defibrinated sheep blood, 25,000 IU of bacitracin, 10,000 IU of polymyxin B, 5 mg of novobiocin and 50 mg of actidione per liter of fluid thioglycollate agar medium. The inoculated plates were held at 37°C for 3 d under a reduced atmosphere. This original selective medium enabled workers to isolate *C. jejuni* from the stools of sick individuals.

This original medium has been modified several times. Recently, Patton et al. (*33*) described a modified Butzler's formula (*23*) comprised of 29.8 g of fluid thioglycollate medium, 30 g of agar, 1,000 ml of distilled water, 100 ml of defribrinated sheep blood, 25,000 IU of bacitracin, 5 mg of novobiocin, 50 mg of actidione, 15 mg of cephalothin and 40,000 U of colistin per liter. Of three selective media com-

pared for efficiency in primary isolation of *C. jejuni* from rectal swabs of animals, recovery rates with the modified Butzler formulation were significantly higher than those obtained with Skirrow's (*39*) and Butzler's original formulation (*23*).

Skirrow's formulation (*39*) consists of 5 to 7% lysed horse blood, 10 mg of vancomycin, 2,500 IU of polymyxin B and 5 mg of trimethoprim per liter of either blood agar base no. 2 or brucella agar. Using this selective medium, under a reduced atmosphere at 43°C, Skirrow isolated *C. jejuni* from 7.1% of 803 patients with diarrhea. No filtering is needed before plating with this selective formulation. Blaser et al. (*2*) modified Skirrow's formula to produce Campy-BAP, the third of the commonly used selective media for isolating *Campylobacter*. To Skirrow's formulation, 2 mg of amphotericin B and 15 mg of cephalothin were added per liter. Cephalothin reduced the normal enteric flora contaminants and amphotericin B inhibited the growth of *Candida albicans*.

In a recent study, selective plates of Skirrow's medium (*8*) modified with 15 mg of cephalothin per liter, Campy-BAP (*3*) and Butzler's (*23*) agar were tested for their sensitivity and selectivity in the recovery of inoculated *C. jejuni* from ground beef (*47*). Campy-BAP medium was the most sensitive and Butzler's medium the most selective. Breakthrough contaminants on these plates were noted and discussed. Stern (*47*) suggested that a combination of both Campy-BAP and Butzler's media would be most useful in the recovery of *C. jejuni* from food sources.

Identification

Plates should be examined at 24, 48 and 72 h for typical colonies of *C. jejuni*. If none is apparent, the plates should be returned to the incubator and microaerobic conditions should be re-established. Typical colonies of *C. jejuni* on selective blood agar plates will be non-hemolytic flat or slightly raised, have an irregular edge or round, and appear gray to pink or tan mucoid (Fig. 2). If the humidity is high within the incubation chamber or the plates are wet, the organism tends to spread in a characteristic water droplet-like fashion. After 48 h of incubation, the colonies are generally 1 to 2 mm in diameter.

Characteristic colonies should be transferred to a wet-mount slide and observed with a phase-contrast microscope. Details of taxonomical criteria may be found in *Bergey's Manual of Determinative Bacteriology (41)*. The most important criterion to consider in identifying *C. jejuni* is its rapid corkscrew, to-and fro-motion. The organism is narrow (0.2 to 0.5μm) and appears in comma, S, gull or spiral shapes (Fig. 3). If an isolate has the characteristic shapes and motion, one should further test for lack of growth under microaerobic conditions at 25°C. Further, *C. jejuni* does not grow under aerobic conditions, is resistant to discs of cephalothin (30 μg), and is often susceptible to discs of nalidixic acid (30 μg) with some notable exceptions (*40*). The organism is capable of growth in 1% glycine, produces both oxidase and catalase, and reduces nitrate to nitrite without further reduction. *C. jejuni* does not grow in 3.5% sodium chloride nor does it ferment glucose. The organism does not produce H_2S in triple sugar iron agar but does pro-

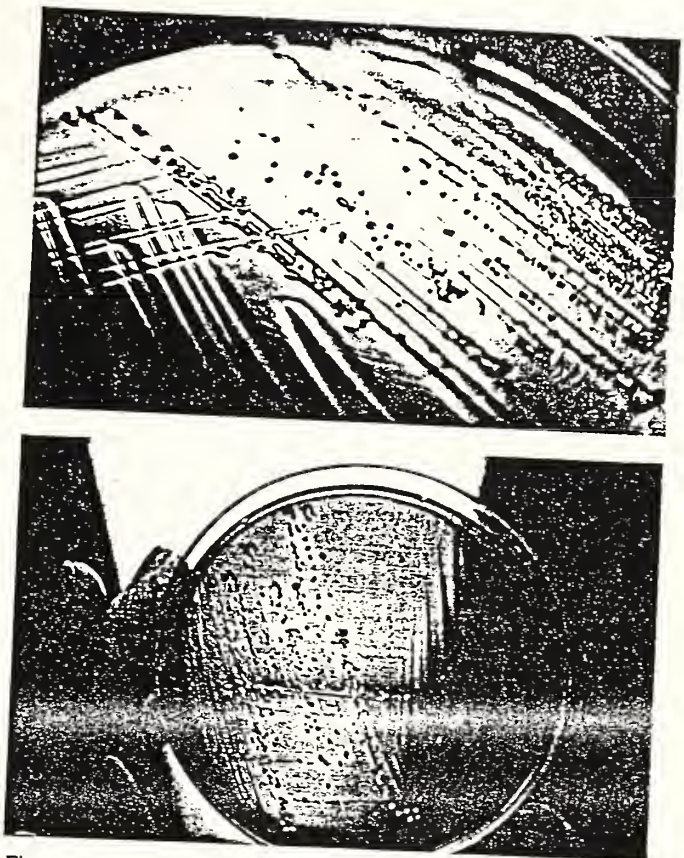

Figure 2. *Characteristic appearance of* Campylobacter jejuni *in pure culture on a selective blood agar plate after 24 h at 42°C under microaerobic conditions.*

Figure 3. *Electron photomicrograph of* Campylobacter jejuni *isolated from a pig carcass and grown overnight in fluid thioglycollate medium at 42°C.*

duce H_2S as detected by the presence of a lead acetate strip over a medium containing 0.02% cysteine-HCl. An excellent basal medium for assessing these characteristics is brucella broth containing 0.16% agar (*16*).

It has been suggested that isolates conforming to the above criteria may further be divided into two separate species, *C. jejuni* and *C. coli (40)*. *C. coli* grows at 30.5°C, resists 2, 3, 5-triphenyltetrazolium chloride and does not hydrolyze sodium hippurate (*18*). This species may be discriminated from *C. jejuni*, which produces the opposite results as *C. coli* does for these three tests. In our hands, the hippurate test is the most reproducible of the three suggested discriminating tests. However, this "discrimination" may not be of critical importance to food microbiologists since both *C. jejuni* and *C. coli* are responsible for human infections.

An abbreviated flow chart is given as a procedure useful in isolating *C. jejuni* from foods (Fig. 4). Some of these methods are new and still need confirmation by other laboratories. By using this protocol, workers in my laboratory have routinely isolated *C. jejuni* from food sources. The recovery method appears to be no more difficult than the well-established procedures currently in use for other foodborne pathogens. Rapid presumptive tests for use by the food industry and in public health laboratories still needs to be developed.

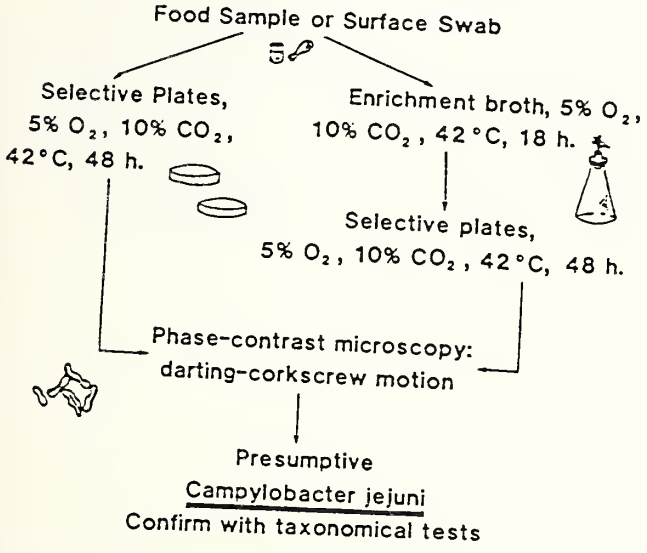

Figure 4. *Flow chart suggesting procedures for the isolation of* Campylobacter jejuni *from foods.*

ACKNOWLEDGMENTS

This paper was presented in part at the 1982 Annual Meeting of the American Society for Microbiology, Atlanta, GA.

The author gratefully acknowledges the superb assistance given by R. Nisley in the preparation of this manuscript.

REFERENCES

1. Anonymous. 1981. Campylobacter infections, 1977-80. Br. Med. J. 282:1484.
2. Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W-L. L. Wang. 1979. Campylobacter enteritis: clinical and epidemiologic features. Ann. Intern. Med. 91:179-185.
3. Blaser, M. J., J. Cravens, B. W. Powers, F. M. LaForce, and W-L. L. Wang. 1979. Campylobacter enteritis associated with unpasteurized milk. Am. J. Med. 67:715-718.
4. Blaser, M. J., P. Checko, C. Bopp, and J. Hughes. 1981. Foodborne *Campylobcter* enteritis. Abstr. Ann. Meet. Am. Soc. Microbiol., Dallas, TX., C 10, p. 264.
5. Brouwer, R., M. J. A. Mertens, T. H. Siem, and J. Katchaki. 1979. An explosive outbreak of *Campylobacter* enteritis in soldiers. Antonie van Leeuwenhoek 45:517-519.
6. Buck, G. E., C. Fojtasek, K. Calvert, and M. T. Kelly. 1982. Evaluation of the CampyPak II gas generator system for isolation of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 15:41-42.
7. Butzler, J. P., and M. B. Skirrow. 1979. *Campylobacter* enteritis. Acta. Paediatr. Belg. 32:89-94.
8. Chan, F. T. H., and A. M. R. Mackenzie. 1982. Enrichment medium and control system for isolation of *Campylobcter fetus* subsp. *jejuni* from stools. J. Clin. Microbiol. 15:12-15.
9. Dekeyser, P., M. Gossuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related vibrio: first positive stool cultures. J. Infect. Dis. 125:390-392.
10. Doyle, M. P. 1981. *Campylobcter fetus* subsp. *jejuni:* an old pathogen of new concern. J. Food Prot. 44:480-488.
11. Doyle, M. P., and D. J. Roman. 1982. Recovery of *Campylobacter jejuni/coli* by selective enrichment. Appl. Environ. Microbiol. 43:1343-1353.
12. George, H. A., P. S. Hoffman, R. M. Smibert, and N. R. Krieg. 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. J. Clin. Microbiol. 8:36-41.
13. Grant, I. H., N. J. Richardson, and V. D. Bokkenheuser. 1980. Broiler chickens as potential source of *Campylobacter* infections in humans. J. Clin. Microbiol. 11:508-510.
14. Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert. 1979. Studies of the microaerophiclic nature of *Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. 25:8-16.
15. Hoffman, P. S., N. R. Krieg, and R. M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. 25:1-7.
16. Holdeman, L. V., E. P. Calto, and W. E. C. Moore (eds.). 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, VA.
17. Hudson, W. R., and T. A. Roberts. 1981. The occurrence of *Campylobacter* on commercial red meat carcasses from one abattoir. Abstracts of the Public Health Laboratory Service International Workshop on *Campylobacter* Infections. University of Reading. p. 34.
18. Hwang, M-N., and G. M. Ederer. 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. J. Clin. Microbiol. 1:114-115.
19. Kaijser, B., and A. Svedhem. 1981. The occurrence of *Campylobacter jejuni* in fresh food and its survival under different conditions. Abstracts of the Public Health Laboratory Service International Workshop on *Campylobacter* Infections, University of Reading. p. 11.
20. Karmali, M. A., and P. C. Fleming. 1979. Application of the Forner principle to isolation of *Campylobacter* from stools. J. Clin. Microbiol. 10:245-247.
21. King, E. O. 1957. Human infections with *Vibrio fetus* and a closely related vibrio. J. Infect. Dis. 101:119-128.
22. Lander, K. P., and K. P. W. Gill. 1980. Experimental infection of the bovine udder with *Campylobacter coli/jejuni*. J. Hyg. 84:421-428.
23. Lauwers, S., M. DeBoeck, and J. P. Butzler. 1978. Campylobacter enteritis in Brussels. Lancet 1:604-605.
24. Levy, A. J. 1946. A gastroenteritis outbreak probably due to a bovine strain of vibrio. Yale J. Biol. Med. 18:243-259.
25. Luechtefeld, N. W., and W-L. L. Wang. 1981. *Campylobacter fetus* subsp. *jejuni* in a turkey processing plant. J. Clin. Microbiol. 13:266-268.

26. Luechtefeld, N. W., L. B. Reller, M. J. Blaser, and W-L. L. Wang. 1982. Comparison of atmospheres of incubation for primary isolation of *Campylobacter fetus* subsp. *jejuni* from animal specimens: 5% oxygen versus candle jar. J. Clin. Microbiol. 15:53-57.
27. Norberg, P. 1981. Enteropathogenic bacteria in frozen chicken. Appl. Environ. Microbiol. 42:32-34.
28. Oosterom, J. 1981. *Campylobacter* outbreak in a military camp: investigations, results and further epidemiological studies. Abstracts of the Public Health Laboratory Service International Workshop on *Campylobacter* Infections, University of Reading. p. 37.
29. Oosterom, J., M. J. G. M. Vereijken, and G. B. Engels. 1981. *Campylobacter* isolation. Vet. Quart. 3:104.
30. Pai, C. H., S. Sorger, L. Lackman, R. E. Sinai, and M. I. Marks. 1979. *Campylobacter* enteritis in children. J. Pediatr. 94:589-591.
31. Park, C. E., and Z. K. Stankiewicz. 1981. A selective enrichment procedure for the isolation of *Campylobacter jejuni* from foods. Abstracts of the Public Health Laboratory Service International Workshop on *Campylobacter* Infections, University of Reading. p. 13.
32. Park, C. E., Z. K. Stankiewicz, J. Lovett, and J. Hunt. 1981. Incidence of *Campylobacter jejuni* in fresh eviscerated whole market chickens. Can. J. Microbiol. 27:841-842.
33. Patton, C. M., S. W. Mitchell, M. E. Potter, and A. F. Kaufmann. 1981. Comparison of selective media for primary isolation of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 13:326-330.
34. Porter, I. A., and T. M.S. Reid. 1980. A milk-borne outbreak of *Campylobacter* infection. J. Hyg. 84:415-419.
35. Robinson, D. A. 1981. Infective dose of *Campylobacter jejuni* in milk. Br. Med. J. 282:1584.
36. Robinson, D. A., and D. M. Jones. 1981. Milk-borne *Campylobacter* infection. Br. Med. J. 282:1374-1376.
37. Rosef, O. 1981. Isolation of *Campylobacter fetus* subsp. *jejuni* from the gallbladder of normal slaughter pigs, using an enrichment procedure. Acta Vet. Scand. 22:149-151.
38. Simmons, N. A., and F. J. Gibbs. 1977. *Campylobacter* enteritis. Br. Med. J. 2:264.
39. Skirrow, M. B. 1977. *Campylobacter* enteritis: a "new" disease. Br. Med. J. 2:9-11.
40. Skirrow, M. B., and J. Benjamin. 1980. "1001" campylobacters: cultural characteristics of intestinal campylobacters from man and animals. J. Hyg. 85:427-442.
41. Smibert, R. M. 1974. *Campylobacter*. pp. 207-212. *In* R. E. Buchanan and N. E. Gibbons (eds.) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins, Baltimore.
42. Smibert, R. M. 1978. The genus *Campylobacter*. Ann. Rev. Microbiol. 32:673-709.
43. Smith, M. V., and P. J. Muldoon. 1974. *Campylobacter fetus* subsp. *jejuni (Vibrio fetus)* from commercially processed poultry. Appl. Microbiol. 27:995-996.
44. Smith, T., and M. S. Taylor. 1919. Morpohology and biology of *Vibrio fetus*. J. Exp. Med. 30:299-311.
45. Stern, N. J. 1981. *Campylobacter fetus* subsp. *jejuni:* recovery methodology and isolation from lamb carcasses. J. Food Sci. 46:660-661, 663.
46. Stern, N. J. 1981. Recovery rate of *Campylobacter fetus* ssp. *jejuni* on eviscerated pork, lamb and beef carcasses. J. Food Sci. 46:1291, 1293.
47. Stern, N. J. 1982. Selectivity and sensitivity of three media for recovering *Campylobacter fetus* ssp. *jejuni* from ground beef. J. Food Safety 4:169-175.
48. Svedhem, A., and B. Kaijser. 1980. *Campylobacter fetus* subspecies *jejuni:* a common cause of diarrhea in Sweden. J. Infect. Dis. 142:353-359.
49. Tanner, E. I., and C. H. Bullin. 1977. *Campylobacter* enteritis. Br. Med. J. 2:579.
50. Taylor, P. R., W. M. Weinstein, and J. H. Bryner. 1979. *Campylobacter fetus* infection in human subjects: association with raw milk. Am. J. Med. 66:779-783.
51. Terhune, C., E. Sazi, N. Kalishman, J. Bobst, B. Bonnlander, J. A. Googins, and P. Williams. 1981. Raw milk-associated campylobacteriosis--Oregon. Morbid. Mortal. Weekly Rep. 30:90-92.
52. Tosh, F. B., G. A. Mullen, and D. E. Wilcox. 1981. Outbreak of *Campylobacter* enteritis associated with raw milk--Kansas. Morbid. Mortal. Weekly Rep. 30:218-220.
53. Turnbull, P. C. B., and P. Rose. 1981. *Campylobacter jejuni/coli* in raw red meats: a public health laboratory service survey. Abstracts of the Public Health Laboratory Service International Workshop on *Campylobacter* Infections, University of Reading, p. 41.
54. Veron, M., and R. Chatelain. 1973. Taxonomic study of the genus *Campylobacter* Sebald and Veron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Veron. Int. J. Syst. Bacteriol. 22:122-134.

The laboratory procedure used at

Russell Research Center to isolate *Salmonella*

PREENRICHMENT (if needed)	-	M - 9 OR BP
ENRICHMENT	-	SC AND TT
PLATING	-	BG SULFA AGAR, MLIA/USDA
SCREENING	-	LIA
CONFIRMATION	-	MICRO ID - SEROLOGY

RRC *Salmonella* procedure

1. Preenrichment is optional. If you want the absolutely most sensitive procedure and are not worried about an extra day's work, or if the product you are sampling has been stressed in any way then I would recommend preenriching in either a 1% buffered peptone or minimal salts(M-9) medium for 18-24 hr.

2. Enrichment--If you are direct enriching raw poultry without a preenrichment step then I would recommend selenite cystine broth. If you preenriched first, then I would make 1 to 10 transfers of the preenrichment broth into both selenite cystine (incubate at 35 C for 18-24 hr.) and tetrathionate brillient green or TT (incubate at 42 C for 18-24 hr.). For your most sensitive results it is best to use both enrichments because you will ocassionally find some serotypes of salmonellae which will be inhibited by one of the enrichments.

3. Selective Plating--We recommend using BG sulfa and MLIA/USDA (see enclosed manuscript) as our two primary plating media. Streak plates for isolation and incubate for 24-48 hr. at 35 C.

4. Screening--Stab and streak lysine iron agar slants and incubate overnight with the caps loosely fastened. Look for typical purple/purple with blackening along the stab line.

5. Confirmation--We use Micro ID or API for biochemical characterization and poly O and Poly H serology for biochemical confirmation.

Product Sampling

Whole carcarsses--We recommend rinse sampling with 100 ml sterile water (allows rehanging carcasses on processing line) and then adding 10x concentrated preenrichment or enrichment to give a single strength solution.

Parts--Use a similar approach.

Communited product--Make a 1 to 10 dilution of your product into a stomacher bag filled with the appropriate medium and then stomach for 30 sec.